Docket No.: 023004.0103X1US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reexamination Application of:
Michael W. Graham et al.

Application No.: 90/007247 — Confirmation No.: 6310

Filed: October 4, 2004 — Art Unit: 1639

For: GENETIC CONSTRUCTS FOR DELAYING OR REPRESSING THE EXPRESSION OF A TARGET GENE — Examiner: B. M. Celsa

DECLARATION UNDER 37 C.F.R. § 1.131

Customer Window, MS Amendment
U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

Dear Sir:

I, Michael Graham, Ph.D., declare as follows:

1. I am a named inventor of the subject matter in the above-identified reexamination. I am a resident and citizen of Australia. During the period of December 1997 through the filing of the priority document for the patent under reexamination, I was a research scientist in Australia. During this period Robert Rice and Margaret Bernard were under my direction and supervision.

2. I have reviewed the above-identified reexamination, including the present claims. As I understand it, the presently claimed subject matter is generally directed to genetic constructs that are capable of delaying, repressing or otherwise reducing the expression of a target gene in an animal cell, as well as methods for using these constructs and animal cells comprising these constructs. I understand that the presently claimed constructs comprise at least one structural gene sequence placed operably in a sense orientation under the control of a

promoter and at least one structural gene sequence placed operably in an antisense orientation under the control of a promoter, where the structural gene sequences comprise a nucleotide sequence which is substantially identical to at least a region of a target gene, and where

a. the multiple structural gene sequences are placed operably under the control of a single promoter sequence, where optionally the structural gene sequences in sense and antisense orientations are spaced from each other by a nucleic acid stuffer fragment; or

b. the structural gene sequences in sense and antisense orientations are each placed operably under the control of individual promoter sequences.

3. I am aware of the rejections issued in an Office Action mailed January 24, 2007, in the pending reexamination. I understand that a rejection in the Office Action was based on the teachings of Fire U.S. Patent No. 6,506,559 (the "Fire reference"). The Fire reference was filed in late 1998, and claims priority to U.S. Serial No. 60/068,562 (the "Fire priority application") filed December 23, 1997, less than a year before the effective filing date of the patent under reexamination in the United States. It is my understanding that to show prior invention, the Examiner is requiring that I provide evidence of conception prior to the date of filing of the Fire priority application and then the Examiner is requiring that I show diligence from just before the filing date until reduction to practice or constructive reduction to practice of my own invention.

4. Exhibit 1 is a copy of laboratory notebook pages showing my preliminary work in plants. My early work on genetic constructs for reducing expression of a target gene was in plants and I spent significant amounts of time trying to produce such constructs. I consider this work important to my present invention because the layout of the constructs in plants was the basis for my later attempt in animals. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. I conceived the subject matter of the presently claimed invention prior to December 23, 1997. Evidence for conception before the date of the Fire priority application includes laboratory notebook pages 107 – 108, which show one

of my first attempts to make a genetic construct which was designed to express both sense and antisense RNAs from multiple copies of a nucleotide sequence under the control of a promoter. Page 108 in particular shows my drawing of a "Hairpin GUS" construct that includes two copies of a structural gene sequence in the antisense and sense orientation, expression of which was driven by a single promoter. Laboratory notebook pages 110 – 121 show my additional experiments to build constructs with structural gene sequences in a sense and antisense orientation. Laboratory notebook pages 130 – 32 show experiments where I attempted to make expression cassettes containing two promoters designed to express separate sense and antisense RNAs. Laboratory notebook pages 138, 145, 147, 150, 151, 159, 169, 175, 181-192, 206, 216, 229 and 266 show experiments where I continued to try and create genetic constructs expressing separate sense and antisense RNAs. Laboratory notebook pages 138, 145, 150, 153, 158, 165, 168, 172, 175, 182, 185, 195, 197, 198, 200, 210, 227, 229, 240 and 254 show experiments where I developed constructs in which the structural genes were orientated in a sense and antisense orientation, some of which were controlled by separate promoters. I understand that this is evidence of a conception of genetic constructs of the same type as those of the claimed invention earlier than the priority date of Fire rather than the earliest conception of the claimed invention, which occurred before these notebook entries.

5. Further evidence of conception before the date of the Fire priority application includes the June 6, 1994 letter from CSIRO to John Slattery, as indicated in Exhibit 2. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. In this letter, my employer at the time, CSIRO, requested from Mr. Slattery an opinion on the patentability of my new constructs that I believed would "be useful in mammalian systems", as I indicated on the last page of the attachment. Attached to the letter is my idea to create constructs such as the subject matter in the above-identified reexamination. For instance, the second figure, Case 2, is a construct comprising a single promoter that transcribes two structural gene sequences in an inverted repeat to form a transcript with a "hairpin" structure, where the inverted repeat is not separated by a stuffer fragment. This figure shows my idea of making a construct like the construct of Claim 3. The third figure, Case 3, is a construct comprising two copies of a structural gene sequence, where one copy is placed operably in the sense

orientation under the control of an individual promoter and the other copy is placed operably in the antisense orientation under the control of a separate promoter. Case 3 therefore shows my idea of making the construct of Claim 4 and indicates I had conceived of this invention on or before June 6, 1994.

6. Exhibit 3 is an early outline for a provisional patent application which I prepared on August 8, 1995. In this draft, I discuss decreasing gene expression in animals by use of novel transgene designs. This Exhibit was previously submitted in the June 12, 2006 37 C.F.R. § 1.131 declaration.

7. Exhibit 4 is a draft of an unpublished manuscript which I prepared on June 21, 1996. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. I prepared this article for publication in a journal to try and demonstrate the widespread existence of an RNA degradative system in plants and other organisms based on a re-interpretation of the literature that existed at that time. While genetic constructs were not described in this document, their development was based on ideas presented therein, specifically I wished to design constructs to switch this RNA degradative system on more efficiently in transgenic organisms. I discussed evidence that this RNA degradative system existed in animals in the section titled "Post-transcriptional gene inactivation in other taxa" and believe this document demonstrates my thinking at that time, namely the types of constructs that might work in plants would also work in animals.

8. Exhibit 5 is a draft of a proposal I prepared on November 29, 1996, proposing that genetic constructs for gene silencing would prove effective in animals. This proposal was important because budgetary limits at Benitec (then called Ag-Gene and my employer at that time) inhibited my ability to conduct all of the research I intended for target gene inactivation. In this proposal, I discuss the genetic constructs I previously created for plants and how I wanted to create "multiple gene constructs, the use of direct and inverted sequences and the design and use of RNA stabilizing sequences" to decrease gene expression in animals. When our funding increased, we promptly hired Robert Rice to work on post-transcriptional gene silencing in animal cells.

9. Exhibit 6 shows further evidence of my conception before the date of the Fire priority application. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. Laboratory notebook pages 52 – 55, which are from May 1997, show how I conceived a genetic construct design to express hairpin RNAs where the construct had a stuffer fragment inserted between the two copies of the nucleotide sequence, where one of the sequences was in a sense orientation and the other sequence was in an antisense orientation relative to the promoter. I consider this Exhibit important because previously I had difficulty creating inverted repeat constructs without a stuffer fragment due to instability of such constructs in *E. coli*. The insertion of the stuffer fragment between the inverted repeat sequences allowed me to readily make such constructs, and as such I planned to incorporate this idea into the genetic constructs for reducing expression of animal genes. Thus, compared to constructs without a stuffer fragment, the inverted repeat constructs with a stuffer fragment were superior.

10. I understand the Examiner would like to see the diligence to reduce my invention to practice between the Fire priority date of December 23, 1997, and the '099 patent priority date of March 20, 1998. As such, I detail below the events that occurred. To assist the Examiner, I also include a calendar of December 1997, and January – March 1998.

December 1997

Su	Mo	Tu	We	Th	Fr	Sa
	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
21	22	23	24	25	26	27
28	29	30	31			

7:◐ 13:○ 21:● 29:●

January 1998

Su	Mo	Tu	We	Th	Fr	Sa
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	31

5:● 12:○ 20:◑ 28:●

February 1998

Su	Mo	Tu	We	Th	Fr	Sa
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28

3:● 11:○ 19:◑ 26:●

March 1998

Su	Mo	Tu	We	Th	Fr	Sa
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

5:● 12:○ 21:◑ 27:●

11. When Ag-Gene funding increased in late 1997, we promptly pursued hiring Robert Rice to work on preparing gene constructs for gene silencing in animal cells, corresponding to the designs I had conceived. From what I recall, we at Ag-Gene started discussing hiring Dr. Rice in October 1997. We wanted to work with Dr. Rice because he had extensive experience in a range of molecular biological techniques and plasmid design and construction. Dr. Rice's thesis topic was eukaryotic evolution and studying eukaryotic divergence using ribosomal RNA sequence data and secondary structure remodeling. As such, Dr. Rice also had experience with use of computers for systematic / bioinformatics analysis of DNA / RNA sequences.

12. On December 8, 1997, I decided to target the polymerase gene of the bovine enterovirus (BEV) as an exemplary target gene in animal cells. This gene was chosen because it could be easily determined whether the expression of constructs based on the gene had an effect on viral replication in animal cells. Specifically, since infection of Mabin Darby (MDBK) cells with BEV normally kills them, we could therefore determine whether expression of constructs in transformed cells might inhibit viral replication simply by determining whether such transformed cells show prolonged survival following challenge with the virus under standardised conditions. Further, we knew that the BEV polymerase may be amplified using the polymerase chain reaction or alternatively, isolated using standard hybridisation techniques. With the assistance of Margaret Bernard ("Ms. Bernard"), I printed out the sequence of the polymerase gene of BEV, see Exhibit 7, page 2. (indicating the sequence was

printed at 3:13pm on December 8, 1997). Again with the assistance of Ms. Bernard, I designed a pair of oligonucleotide primers to amplify a region of the BEV gene. These primers, designated BEV-1 and BEV-2 (pages 2-3 of Exhibit 7) were ordered by Ms. Bernard from a commercial supplier under my instruction on December 9, 1997. (*See, Id.* at 1, lower entry: the notation the primers were ordered December 9, 1998 is in error; they were ordered December 9, 1997 as evidenced by their entry on the notebook page of December 9, 1997 and their use on January 6, 1998). These primers were available for use by Ms. Bernard on January 6, 1998. We continued with BEV as a target gene all the way to actually practicing the invention, as can be seen in the figures in the patent application that we filed.

13. On or about December 8, 1997, I mentioned to Ms. Bernard that as soon as possible she would be devoting a greater amount of her time for work on a project with the new Research Scientist, Dr. Rice, in preparing the gene constructs for the animal target gene, in particular the constructs targeting BEV.

14. Dr. Rice arrived to commence employment on the "animal project" on December 21, 1997. On that day or the day after, I met with Dr. Rice and described to him in detail the types of constructs that I had envisaged for reducing expression of a target gene. The first type of construct was an inverted palindrome construct without a stuffer fragment. Claim 3 of my patent under this reexamination is to the inverted palindrome construct without the stuffer fragment and claim 7 is to a method of using the construct. The second type of construct was an inverted palindrome construct with a stuffer fragment. Claim 5 is to the inverted palindrome construct with the stuffer fragment and claim 9 is to a method of using the construct. Finally, I wanted to make a construct with two copies of a gene sequence where each copy was under the control of a separate promoter. Claim 4 is to this type of construct and claim 10 is to a method of using this construct. On the days following, I had further discussions with Dr. Rice about these types of constructs.

15. The laboratory facilities of Ag-Gene were located at the Queensland Agricultural Biotechnology Centre (QABC), an operational centre of the Queensland State Government's Department of Primary Industries. The Queensland State Government provided paid leave

for Christmas day (December 25), Boxing Day (December 26) and New Year's Day (January 1). Further, the Queensland State Government mandated that all State Government employees do not work on the days between December 26 and January 1. As such, the QABC laboratories and offices were closed from December 25, 1997 to January 1, 1998.

16. Dr. Rice and I met several times between December 21, 1997 and mid-January 1998 to discuss cosuppression in animal cells and the types of DNA constructs we wanted to prepare. We decided to build a range of constructs with the following structures: linear repeats, that is constructs containing a block of repeated DNA sequences in sense or in an antisense orientation; inverted repeats, that is constructs containing two inverted DNA sequences either with or without a DNA spacer sequence inserted between the inverted sequences; and a construct with two promoters expressing a sequence in the sense and antisense format.

17. From January 1998 to March 1998, Dr. Rice designed approximately 40 plasmid constructs. Exhibit 8 contains approximately 35 plasmid constructs he designed, most of which are also found as figures of the '099 patent.

18. When Ms. Bernard returned from her Christmas vacation on January 5 or 6, 1998, Dr. Rice and I informed her that we wanted her to prepare certain BEV constructs. We described the kind of constructs we wanted, namely the three constructs discussed above in paragraph 14. Ms. Bernard, with my assistance, was to start preparing the BEV constructs. *See*, Exhibit 7 at page 1. In the meantime, Dr. Rice was to use a computer program to design further genetic constructs. Dr. Rice and I explained to Ms. Bernard that the overall aims of the experiments were to "use Bovine enterovirus as a model system to study cosuppression in mammalian cells," which Ms. Bernard recorded in her laboratory notebook at page 2. Ms. Bernard took further notes from our talk, writing down the polymerase gene from BEV was to be used as the sequence for the animal constructs. *Id.* Ms. Bernard states in her notes that once the constructs were prepared, she was going to "transfect mammalian cell line with constructs, probably using the Mabin Darby Bovine Kidney (MDBK) endothelial cell line." *Id.* The cells would then be challenged with BEV. *Id.* Ms. Bernard then describes the initial constructs. *Id.*

19. The first construct to be made was a BEV polymerase-GFP gene fusion in the vector pEGFP-N1. *Id.* In this arrangement, the CMV promoter of pEGFP-N1 lay upstream of the BEV sequence, while the EGFP sequence was placed downstream of and joined to the BEV sequence. Both the BEV and EGFP sequences were designed to be transcribed conjointly by the CMV promoter. The GFP domain was to be used as a marker to indicate BEV-pol positive cells lines and determine whether cosuppression could be detected by transient transfection of BEV-pol positive cells with GFP cDNA. The next construct was similar to the BEV polymerase-GFP fusion construct above, except that the EGFP sequences would be removed and only the BEV sequence would be transcribed from the CMV promoter. *Id.* The next construct describes the use of double promoter constructs (*i.e.*, having two promoters) with the BEV sequence being expressed in sense and antisense format.

20. The January 7, 1998 entry demonstrates Ms. Bernard was cloning the BEV polymerase gene into the carrier plasmid vector pCR2.1. *Id.* at pages 2 – 7. I planned to have her to clone the BEV polymerase gene into pCR2.1, which was the first step of making the BEV polymerase-GFP fusion in the vector pEGFP-N1. Once the BEV polymerase-GFP fusion was in the pEGFP-N1 vector, we planned to use a BglII/BamHI cloning strategy that would result in two alternative fusion constructs where the BEV gene sequence would be cloned in the sense or antisense orientation. Dr. Rice and I believed that once we had the two fusion constructs, we could easily insert the second copy of the BEV gene sequence in the sense and/or antisense orientation into the constructs. This was an element in making the constructs we later claimed in the '099 patent.

21. The primers BEV-1 and BEV-2 were used to PCR amplify the BEV polymerase gene sequence, corresponding to a DNA fragment of about 1.4 kilobases. We then cloned the PCR product into the pCR 2.1 plasmid vector. *Id.*

22. The January 8, 1998 entry demonstrates Ms. Bernard continued the work of January 7, 1998. *Id.* at page 8.

23. The January 9, 1998 entry demonstrates Ms. Bernard continued the work of January 7, 1998. *Id.* at pages 8 – 9.

24. January 10, 1998 was a Saturday and the laboratory was closed.

25. The January 11 and 12, 1998 entry demonstrates Ms. Bernard took steps to grow the clones obtained for the invention. *Id.* at page 9.

26. The January 13, 1998 entry demonstrates Ms. Bernard took further steps to clone BEV into the PCR2.1 and pEGFP. *Id.* at page 10. Further, Ms. Bernard describes how she validated the successful cloning of the BEV polymerase gene sequence into pCR2.1 and confirmed this by endonuclease restriction mapping. *Id.*

27. The January 14 – 16, 1998 entry demonstrates Ms. Bernard took steps to the make the BEV polymerase-GFP fusion in the vector pEGFP-N1. *Id.* at pages 11 – 14. Specifically, Ms. Bernard used a *Bg*III/*Bam*H1 cloning strategy that resulted in two alternative fusion constructs where the BEV gene sequence was cloned in the sense or antisense orientation. *Id.* As previously mentioned, the BgIII/BamH1 cloning strategy that results in two fusion constructs which could be used to easily insert the BEV gene sequence in the sense and/or antisense direction into other constructs.

28. January 17 – 18, 1998 was a Saturday and Sunday and the laboratory was closed.

29. The January 19 – 20, 1998 entry demonstrates Ms. Bernard continued the work of January 14 – 16, 1998. *Id.* at pages 14 – 16.

30. The January 21- 23, 1998 entry demonstrates Ms. Bernard used PCR to check for the presence of the BEV insert. *Id.* at pages 16 – 19. Ms. Bernard drew three diagrams depicting the location of the primers and the expected orientation of the BEV DNA sequence for each PCR product. *Id.* Unfortunately, Ms. Bernard encountered problems and the results were not as expected. *Id.* at page 17. We discussed the matter and agreed that she should try to clone the BEV polymerase gene sequence into the pEGFP-N1 again.

31. The January 21, 1998 entry demonstrates Dr. Rice used a software program to finalize his computer designs of pCR.Bgl.GFP.Bam, pCMV.Virus and pCR2.1, which I believed important to continue development. *See*, Exhibit 8 at pages 1 – 3.

32. As the January 22, 1998 entry demonstrates, Dr. Rice finalized the designs of the constructs pCMV.BEV.2, pCMV.BEVnt, pCMV.BEV.GFP.VEB, pCMV.VEB, pEGFP.BEV.1, pCMV.BEV.VEB, and pCMV.BEVx2 which I believed important to continue development of the invention. *See*, Exhibit 8 at pages 4 – 10. Dr. Rice and I were pleased with these designs. The idea we had was that once Ms. Bernard cloned the BEV polymerase gene sequence into the pEGFP-N1, we could construct pCR.BEV.2. Construction of the pCR.BEV.2 was important to reducing the invention to practice because it could be used to form the constructs we had conceived corresponding to our claims.

33. For example, in one plan we wanted to sub-clone the BEV sequence from the pCR.BEV.2 in the antisense orientation, thus producing the plasmid, pCMV.BEV.VEB. The pCMV.BEV.VEB construct comprises an inverted palindrome of BEV under the control of one promoter. As such, this construct would fall within at least claim 3. This construct is also presented schematically as Figure 14 of the '099 patent. We also wanted to make the above plasmid pCMV.BEV.GFP.VEB. This plasmid comprises an inverted palindrome of the BEV sequence under the control of one promoter with GFP as a stuffer fragment. As such, this construct would fall within at least claim 5. To make this plasmid, we would subclone the GFP from pCR.Bgl.GFP.Bam into pCMV.BEV.2 to produce pCMV.BEV.GFP. We then planned to insert the second BEV sequence in an antisense orientation. The resulting plasmid, pCMV.BEV.GFP.VEB, is presented schematically as Figure 15 of the '099 patent.

34. January 24 and 25, 1998 was a Saturday and Sunday and the laboratory was closed.

35. The January 26 – 28, 1998 entry demonstrates Ms. Bernard again attempted to clone the BEV polymerase gene sequence into pEGFP-N1. Exhibit 7 at pages 20 – 24.

36. From January 29 – February 1, 1998, the transformed cells were allowed to grow.

37. The February 2, 1998 entry demonstrates that the results of the transformation were analyzed and new ligations were set up. *Id.* at pages 25 – 26. Ms. Bernard again encountered problems. As page 25 of Ms. Bernard's notebook indicated, I discussed the results with her, and recommended she try again but instead amend the method. As such, Ms. Bernard again set up experiments to clone the BEV polymerase gene sequence into pEGFP-N1. *Id.* at page 26.

38. Ms. Bernard allowed the DHSα chemically competent cell grow on February 3, 1998. *Id.* at page 37.

39. The February 4 – 6, 1998 entry demonstrates ligations were transformed into the DHSα chemically competent cells. *Id.* at pages 27 – 30. The transformants were then PCR screened. *Id.* at page 30.

40. February 7, 1998 was a Saturday and the laboratory was closed.

41. The February 8 – 11, 1998 entry demonstrates Ms. Bernard's experiments continued. *Id.* at pages 31 – 36. We were pleased to find that Ms. Bernard succeeded in obtaining a fusion clone. *Id.* at page 33. As such, Ms. Bernard went on to sequence the fusion clone to confirm the sequence was in the clone. *Id.* at page 36. Further, Ms. Bernard hand drew a diagram depicting the location of the primers and expected orientation of the BEV-GFP sequence. *Id.*

42. February 14 – 15 was a Saturday and Sunday and the laboratory was closed.

43. Now that we had prepared the fusion clone, we were ready to take the next step. The February 17, 1998 entry demonstrates Ms. Bernard started cloning four new constructs, namely the constructs pCR.BEV.2, pCR.BEV.3, pCR.BamGFPBglII, and pCMV cass. *Id.* at pages 37 – 39. pCR.BEV2 was a construct comprising BEV-pol that could later be used to prepare expression constructs in a sense orientation, or alternatively in an antisense orientation. As previously stated, pCR.BEV2 was an element of reducing the invention to practice because it could be used to form the constructs of our claims, including the

pCMV.BEV.VEB construct which comprises an inverted palindrome of BEV under the control of one promoter. The pCMV.BEV.VEB construct falls within at least claim 3. This construct is also Figure 14 of the '099 patent. pCR.BEV2 was also used to make the plasmid pCMV.BEV.GFP.VEB, which contained an inverted palindrome of BEV under the control of one promoter with GFP as a stuffer fragment. As such, this construct would fall within at least claim 5. Ms. Bernard also started to clone pCR.BEV3, a construct comprising an untranslatable BEV-pol. Ms. Bernard also started to clone pCRBamGFPBglII, which is a construct comprising a stuffer for use in interrupting BEV-pol sense and BEV-pol antisense in a hairpin construct. The EGFP sequence was selected as a stuffer because it would be useful for determining whether the stuffer could mediate post transcriptional gene silencing. The GFP is flanked by the BamH1 and BglII restriction sites, so the GFP would be easy to remove. We planned to use this in our constructs that contained an inverted palindrome with a stuffer, such as pCMV.BEV.GFP.VEB discussed above. Ms. Bernard also started to clone pCMV.cass, which is plasmid pEGFP-N1 except that the EGFP gene sequence has been removed. We chose pCMV.cass as a basic plasmid expression cassette for future clones, and to later make constructs such as pCMV.BEV.SV40L.VEB, which comprises a BEV polymerase placed in the sense orientation to one promoter and another BEV polymerase placed in the antisense orientation to another promoter.

44. The February 18 – 20, 1998 entry demonstrates Ms. Bernard continued her work toward making a BEV polymerase-GFP fusion construct. *Id.* at pages 40 – 41.

45. February 21 and 22 were Saturday and Sunday and the laboratory was closed.

46. The February 23 – 24, 1998 entry demonstrates Ms. Bernard continued the experiments of the previous week. *Id.* at pages 42 – 45. Notably, she identifies the putative fusion clone (#61). *Id.* at page 44.

47. The February 25, 1998 entry demonstrates Dr. Rice designed the construct of pCMV.Lac, the diagram of which is figure 25 of the '099 patent. Exhibit 8 at page 11. Dr. Rice also designed the construct of pCMV.LAC1.pla. *Id.* at page 12.

48. On February 26, 1998, under my and Dr. Rice's direction, Ms. Bernard started to clone pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl by setting up PCR to amplify fragments for the new constructs. Exhibit 7 at page 46. As previously mentioned, I wanted to obtain pCR.BEV2 to make the constructs of our claims, including the plasmid pCMV.BEV.VEB construct, which comprises an inverted palindrome of BEV under the control of one promoter, and the plasmid pCMV.BEV.GFP.VEB, which contained an inverted palindrome of BEV under the control of one promoter with GFP as a stuffer fragment. These constructs correspond to at least claims 3 and 5, respectively.

49. On this same day, Dr. Rice designed the construct of pCMVLac1.OPRSV1.cass, the diagram of which is Figure 26 of the '099 patent. Exhibit 8 at page 13. On this same day Dr. Rice also designed the construct of pCMVLac1.OPRSVL.GFP. *Id.* at page 14.

50. The February 27, 1998 entry demonstrates Ms. Bernard continued her cloning of pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl. Exhibit 7 at page 47. On this same day, Dr. Rice designed the constructs of pCMVLac1.OPRSV1.GFP.cass and pCMV.TYRLIB, the diagrams of which are figures 27 and 24, respectively, of the '099 patent. Exhibit 8 at pages 15 – 16. Dr. Rice also designed the construct pCMVLac.OPRSVL.GFP.TYR. *Id.* at page 17.

51. February 28 and March 1, 1998 were Saturday and Sunday and the laboratory was closed.

52. The March 2, 1998 entry demonstrates Ms. Bernard continued her work to clone the constructs of pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl. Exhibit 7 at page 48. On this same day, Dr. Rice designed the construct of pCMV.TYR, the diagram of which is figure 23 of the '099 patent. Exhibit 8 at page 18.

53. The March 3 – 5, 1998 entry demonstrates Ms. Bernard continued her work to clone the constructs of pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl. Exhibit 7 at pages 49 – 52. Notably, Ms. Bernard confirmed the sequence of the clone on page 50 of the laboratory notebook and the PCR screened the clones on page 51 – 52.

54. The March 5, 1998 entry demonstrates Dr. Rice designed the constructs of pCMV.O.SV40L.BEV, pCMV.O.SV40L.VEB, pCMV.BEV.SV40L.O, pCMV.BEV.SV40L.R, pCR.BEV.1, pCR.BEV.2, pCR.BEV.3, pCR.SV40L, the diagrams of which are figures 17, 18, 16, 22, 6 – 8 and 4, respectively, of the '099 patent. Exhibit 8 at pages 19 – 22, 24 – 29. On this same day, Dr. Rice also designed the construct of pCR.Bgl.GFP.Bam. *Id.* at page 23.

55. The March 6, 1998 entry demonstrates Ms. Bernard ligated the amplified fragment into pPCR 2.1 to obtain pPCR2.1 EGFP. Exhibit 7 at pages 53 – 54. This was then cut with BamH1 and BglII to provide a fragment, that was used to prepare a hairpin construct pBEV2.EGFP.VEB2.

56. On this same day, Dr. Rice designed the constructs of pCMV.cass, pCMV.SV40L.cass, pCMV.SV40LR.cass, pCMV.BEV.SV40L.BEV, pCMV.BEV.SV40L.VEB, the diagrams of which are figures 2, 5, 21, 19, and 20, respectively, of the '099 patent. Exhibit 8 at pages 29 – 33. On this same day, Rice also designed the construct of pCMV.BEV.SV40L.R.cass, pEGFP.N1MCS. *Id.* at page 34.

57. We were excited about the design of the plasmid pCMV.BEV.SV40L.VEB because this plasmid comprises a BEV polymerase placed in the sense orientation to one promoter and another BEV polymerase placed in the antisense orientation to another promoter. This plasmid therefore is an isolated construct of at least claim 4, and indeed corresponds to Figure 20 of the '099 patent. To make this construct, we planned to make a pCMV.SV40L.cass plasmid by sub cloning pCR.SV40L into pCMV.cass, and then insert the BEV polymerase from Ms. Bernard's pCR.BEV.2 into the sense orientation to make pCMV.BEV.SV40L.O. The BEV polymerase from pCR.BEV.2 would then sub cloned into the antisense orientation into the pCMV.BEV.SV40L.O to make pCMV.BEV.SV40L.VEB.

58. In anticipation of making the pCMV.BEV.SV40L.VEB clone, on March 18, Ms. Bernard started preparing the pCMV.cass construct. Exhibit 7 at page 66.

59. March 7 – 8, 1998 was a Saturday and Sunday and the laboratory was closed.

60. The March 9 – 11, 1998 entry demonstrates Ms. Bernard prepared larger amounts of DNA for mammalian cell transfections, including pEGFP.BEV1 and pEGFP-N1. Exhibit 7 at pages 55 – 60. Further, the MDBK cells were split in preparation for transformation on Monday, March 9, 1998. Exhibit 9 at page 1.

61. The March 11, 1998 entry demonstrates I transfected Mabin Darby Bovine Kidney (MDBK) endothelial cells with the pEGFP.BEV.1 constructs. Exhibit 9 at pages 1 – 2.

62. The March 12 – 13, 1998 entry demonstrates Ms. Bernard and I continued our respective experiments. *Id.* at 3; exhibit 7 at pages 61 – 62.

63. March 14 – 15, 1998 were a Saturday and Sunday and the laboratory was closed.

64. The March 16, 1998 entry demonstrates Ms. Bernard obtained the putative clones for pCR.BEV2 and pCR.BEV3. Exhibit 7 at page 63. On this day, I continued my transfection experiment. Exhibit 9 at page 3.

65. The March 17, 1998 entry demonstrates Ms. Bernard confirmed the clones had the proper insert. Exhibit 7 at page 64. As Ms. Bernard stated, the next experiments were to sequence clones with universal forward and reverse primers. *Id.* On this same day, I conducted kill curves for the Mabin Darby Bovine Kidney cells and started selection of constructs. Exhibit 9 at page 4.

66. From March 18 – 19, 1998, Ms. Bernard confirmed the pCR.BEV2 and pCR.BEV3 clones by sequencing. Exhibit 7 at page 67. Further, Ms. Bernard prepared the pCMV.cass construct. *Id.* at pages 65 – 66, 68.

67. The March 20, 1998 entry demonstrates Ms. Bernard continued with transformation of colonies. *Id.* at page 68. The expression cassette pCMV.cass was later confirmed by sequencing. On this same day, I continued my kidney cell transfection experiments. Exhibit 9 at page 5.

68. After this reduction to practice, I filed a patent application in Australia that was the basis for and was claimed as priority by the patent under reexamination.

69. I declare that all statements made of my own knowledge are true and all statements made on information and belief I believed to be true. I make this declaration with the understanding that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the patent.

Michael Graham

April 24, 2007
Date

EXHIBIT 1

Hairpin NLS

(7) GEM3Z NLS Sma HincII Sma then HincII
Cut 3ml in 200ml ē 4 X

(8)

(6) JKK NLS HincII 4
Cut 3ml in 200ml ē 4

Hairpin GUS

(9) pJKK Kpn Sal Sal then Kpn
(3) (1) X
Cut 10ml in 200ml ē 3

(10) pBS-GUS Kpn I/Sma X
Cut 5ml in 200ml ē Sma Sma then KpnI
(4) (1)

(11) pBS-GUS Sma/Sal X
Sma then Sal
Cut 5ml in 200ml Sma (4) (3)

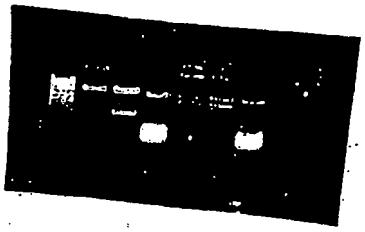
X

GUS → JKK

(12) JKK Kpn/Bam Bam then Kpn
Cut 10ml in 200ml ē Bam (3) (1)

X

(13) pBS GUS Kpn/Bam Bam then Kpn
(3) (1)

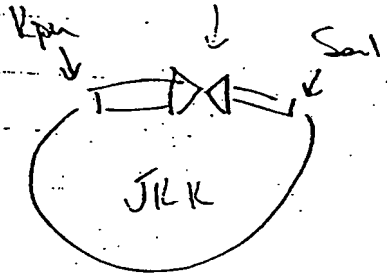


?+9 no good

HincII site in NIa's

	A4	A5
Vector	2ml (9)	2ml (12)
Insert	5ml (10) 5ml (11)	5ml (13)
DDW	5	10
LB	2	2
Ligase	1	1

Descript. HisImpa GUS JKK Ca JKK_GUS



NTh

PAW, Mix High Med Low die 2, → 12
motor Mode

13×15

2→ 9

2+9
more positive

Min 1 + 2 Pools of HAT 7 Nucs

3 -

1-6 Pools of A2

equire Fragments for double DNAs

* pSCHH1T

SCS4 Sma Nhe H3
pHH1T7 Sma Nhe H3

SCS4 Cut 10 ul in 100 ul with Sma (4)

pHH1T7 Cut 3 ul in 100 ul with Sma (4)

Ligations

	J1	J2	J3
ecker	2ul (1)	2ul (2)	1ul (3)
nsert	10ul (4)	5ul (5)	5ul (5)
ddW	5ul	10	11
Buffer	2µl	2	2
Ligase	1ul	1	1

ecipi NheH3 → JKK | NheH3 → pBC | NheH3 → pBS

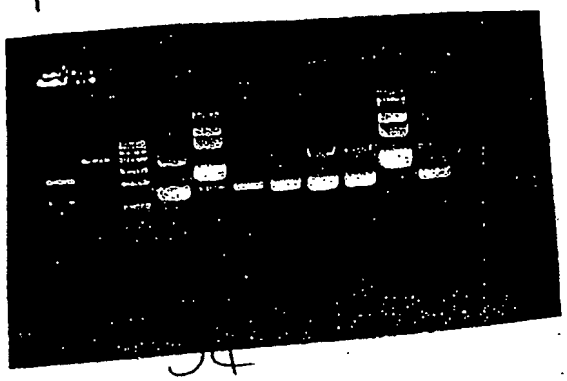
Plate Kan B/W | Plate Cm B/W | Plate Hyg B/W

Have ova BHB 32

Mini-preps

1-9 for H2
10-12 Singles for J3 - picked colonies

Col Purify
of 6,7 SPP1 1-9 uncut



2,7 & 9 maybe OK

Cut 2,7 & 9 ~~EcoRI~~ XbaI/H3

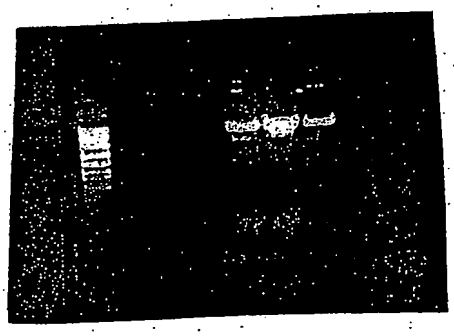
2 ul of 2+7
5 ul of 9

Ligation J4

Vector 2ul 7
Insert 5ul 6
10x buffer 2ul
DiDw 10
Ligase 1

Col

SPP1 10 11 12 , 2, 7 & 9
H3 XbaI/H3



2,7+9 we good!
10,11+12 no DNA.

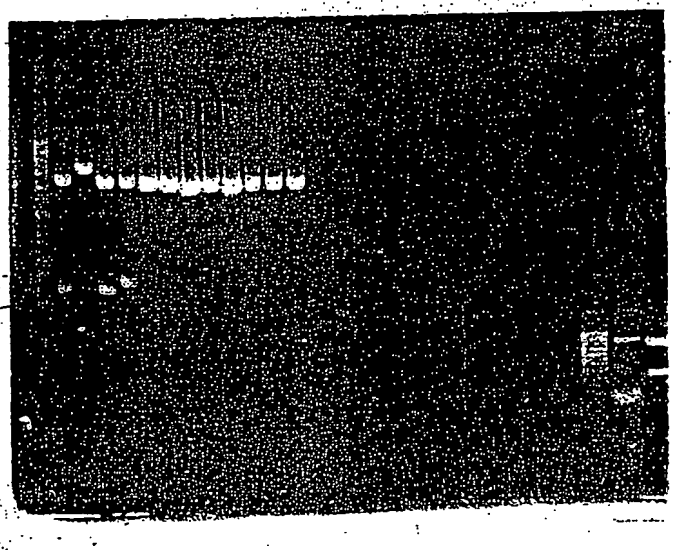
Min 1-6 whites for J3

GUS Assays

1	35S GUS (1) 6	}	Controls
2	Rd1 C GUS		
3	Sh GUS		
4	35S GUS	}	PLRV
5	Rd1 C GUS		
6	Sh GUS		
7	35S GUS		Cold
8	Sh GUS		Cold
9	Rd1C GUS	}	PVY
10	Sh GUS		
11	35SGUS PVY^{O}		
12	35SGUS PVY^{N}		
13	ShGUS 3? PVY^{N}		
14	35SGUS	}	PLRV
15	Sh GUS		
16	Rd1 GUS		

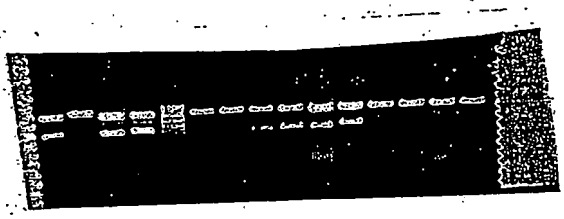
13 Sh GUS PVY^{N}
14 -ve control
15 -ve control

PLRV ones



fH3 is abt ~0.8 kb.

	2nd TG plate Abs/2nd					
			35S 20mg			
			Doc 50m			
			Sh 200mg	PROTEIN	BUFFER	
1	0.43	8.5	35S Cat - 20mg	2.4	97.6	2812
2	0.31	6.1	Doc Cat	16.4	83.6	2601
3	0.31	6.1	Sh Cat	65.6	34.4	- 51
4	0.50	10.0	35S PUV	4	96	2333
5	0.48	9.8	Doc PUV	10.2	89.8	1356
6	0.27	5.3	Sh PUV	75.5	24.5	123
7	0.29	5.8	35S - Cold -	6.9	93.1	3587
8	0.29	5.8	Sh - Cold -	69	31	115
9	0.38	7.6	Doc PUV	13.2	86.8	2207
10	0.26	5.1	Sh PUV	78.4	22.6	88
11	0.85	17.0	35S (PUV)	2.4	97.6	4215
12	0.50	10.0	35S (PUV)	4	96	4395
13	0.42	8.4	Sh (m)	47.6	53.3	133
14	0.36	7.2	-ve (50mg)	m 55.6	44.4	- 28
15	0.39	7.8	-ve (50mg)	m 51.3	48.7	- 29



Min 1-6 H3

H3/R1

Rg1 2/R1

Control

"Cold"

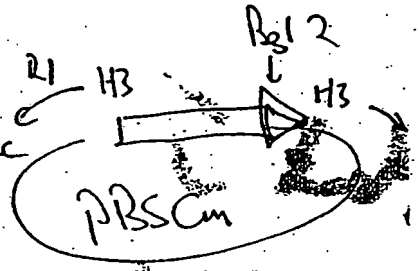
2+3 both good

both the same as.

35S CUS

Cell 2 = 3

3 = 4



CUS

PUV CUS

CHECK J5 R1 Sec Kpu Sna Ba H3

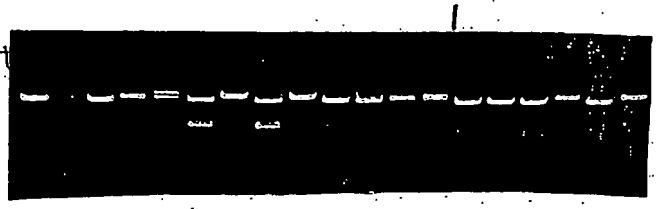
Mains 1-6

J1 Cut H3 (2) MY H3. JKK

Bg1 2/R1 (2)

7-U

J5 Cut R1/H3



Grow 3 as stock

Suspect these O.K

J1 ?

J5 Fine

Cut 3 & 4 as Sequoia Stock

Cleaning!

— Sc4 Bam (blunt) /H3

GEM GZ Nsi (blunt) /H3

Cer ARI 7 SC4 ARI SC4

— JKK SC4 H3/Ba Double header

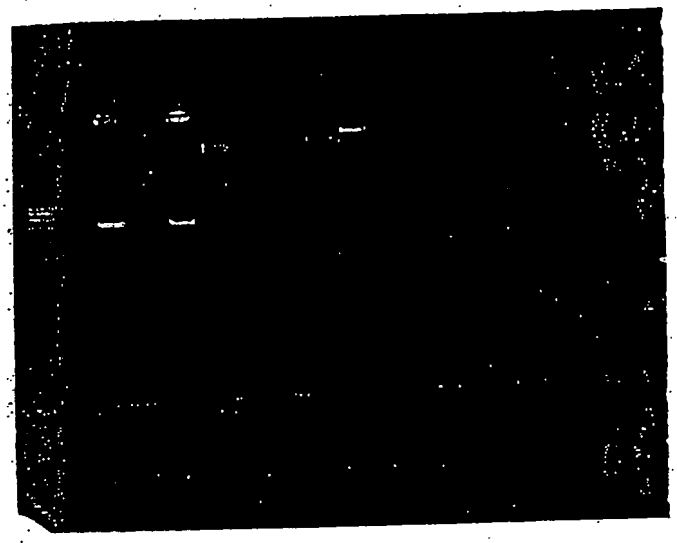
ARI 7 H3/Ba

— JKK SC4 Ba (blunt) / H3

ARI 7 Ba (blunt) / H3 Cla (blunt) /H3

Limbo Stock Cla (blunt)

Cut RISPD1 10, 11, ~~12~~, ~~13~~ 12 15



Ligation

	J6	J7
Vector	Xho (11) Sal (9)	Xho (13)
Insert	Sma (10)	~~Xho (8)~~ Sal (14)
DiH2O	1	1
Xho buffer	2	2
Ligase	1	1
Descript.	↓	
	J6 ↓	J7 ↓

Next
None of these were any good start ...

Repeat (10) (12) (14) (15) (16) (17)

10
~~12~~
13
14
15
} 1-3d

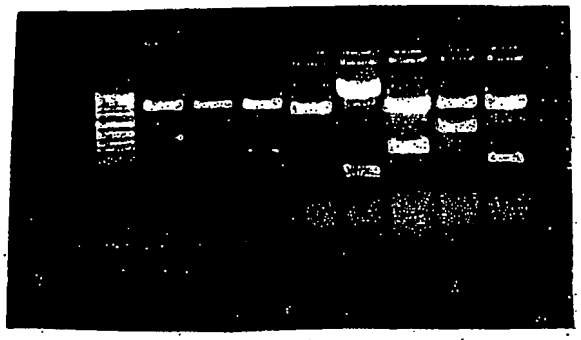
Also ABT7 H3 (18)
pJKK Xho H3 (19)

Xho Sal

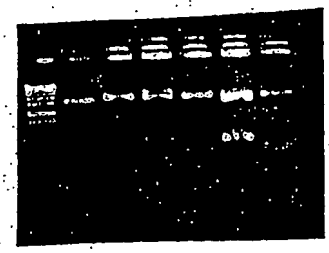
(~~12~~) Sma Xho H3 Cut Sal I Sma (4)
pBSCm Cut Sal with Xho
SL.cass Cut Xho with Sac
Sal I H3

Minis 1, 2 + 3 SalI/KpnI (4)
" NotI (3)

4637 (Rob) BamHI (3) Cut 5ml in 100ml
Check 35S 3' NotI/BamHI (3)
35S Rev NotI/BamHI (3)
pBS SK+ NotI/BamHI (3)



1 - 6



← CHECK 6

Conclusão

SCH connectors.

(1) ADI 7 Δ Not Cut 5ml ∈ Not I (3) 10^{40}

(2) Not Other Cla pBC. SCSH (3 Other 4) 10^{20}
Cut 5ml

(3) Not Other Cla pBC. 355 3' (3 Other 4) 10^{20}

(4) Not /Xhe BC. SCSH Cut 5ml in 3 10^{40}

(5) Not / Xho BC. 355 3' Cut 5ml in 3 10^{40}

(1) + (2) + (3) , (1) + (4) + (5) .

Intermediate [illegible]

(6) Δ BC. LT22 & 5ml Ban/Cla in 2 10^{40}
Than blunt ∈ T4 + recir.

(6) 8 7ml DDW 1^{45}
10ml 10×bulbs
1ml 5mm OWTI's
2ml T4 ligas

	Q1	Q2	Q3
Decks	2ml (1)	2ml (1)	1ml (6) blanket
Invert 1	4ml (2)	4ml (4)	—
2	4ml (3)	2ml (5)	—
Oxiligas	2ml	2ml	2
Ligas	1	1	1
DDW	7	9.	16

ADI 7. SCSH

ADI 7. SCSH
2 ?

Δ BAM/CLA
PBC. LT22

• Cover dots

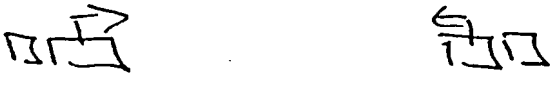
(c) 

(7) ApaI / BI BC. LT22 (2)
Cut 5ul in buffer 2 2h

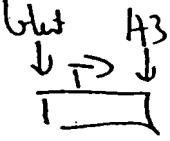
(8) Not / BI BC. 355 3'
Cut 5ul in buffer 3 2h

(9) ApaI / Not1 pBS
Cut 5ul ApaI in buffer 2 2h
Then cut Not1 in buffer 3

Ap BLW x 2

(D) 

(16) JVK SCS4 Cut 5ul
KpnI ~~(blunt)/H3~~ (1)



* Then blunt
Then H3 (2)

87 DPW
10ul 2
3ul H3

Cu 12

(11) HincII / HindIII Cut 5ul buffer 2 2h
PBC. LT22

(12) PBC/BTS Alt 7 Not (blunt) / H3
Alt 7 Not 3ul in buffer 3 2
* Blut
H3 in buffer 3

(19) 4639 Bam I Sma

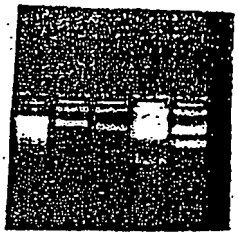
Cut 5 ul Bam I Sma (4)

(20) JKL Nla^s Sma I Bam (4)

Cut 5 ul Bam I Sma

Analytical cuts

- 4639 Bam
- 4639 Sma



(19) + (20)

- JKL Nla^s H3
  H3 I Bgl 2
  Bgl 2
  Sma
  Bam

(17) + (18)

Q & 10

Vecteur 1 µl
Insert 5 µl
DDW 1 µl
10x ligase 1 µl
Ligase 1 µl

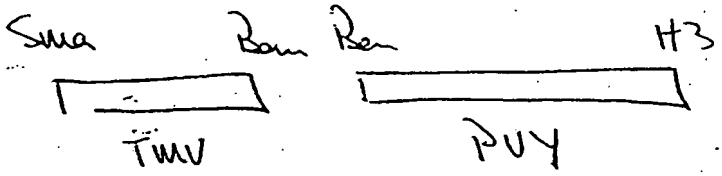
(21) PBC PVY H3

- Bam
- Bam I Bgl 2
- H3

(22)

Clone PUY TMV pairs

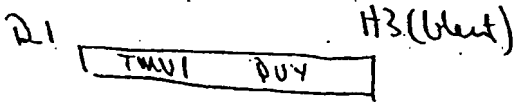
(1)



(2) Pick up

into SmaI/H3 JKK
Both orientations behind pBC.GUS

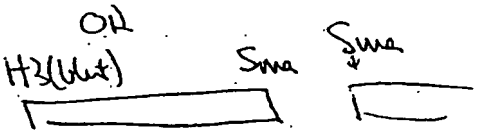
(a)



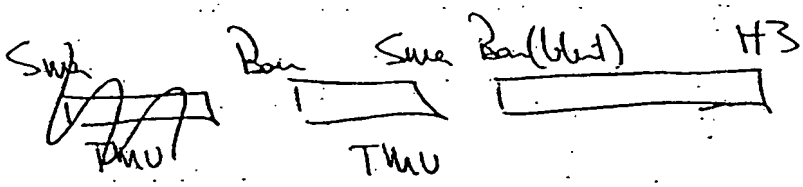
into R1/SmaI pBC.GUS

OR

(b)

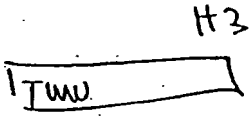


(2)



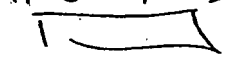
into BamHI/H3 JKK

(a)



⇒ R1/SmaI pBC.GUS

(b)



⇒ R1(blunt) BamHI pBC.GUS

(k) JKK Ban | Sme

Cut 10 ml Ban | Sme 4

10 JKK
10B4
74 DDu
3 Ba
3 Sme

~~(7) JKK~~

(7) 4637 Ban

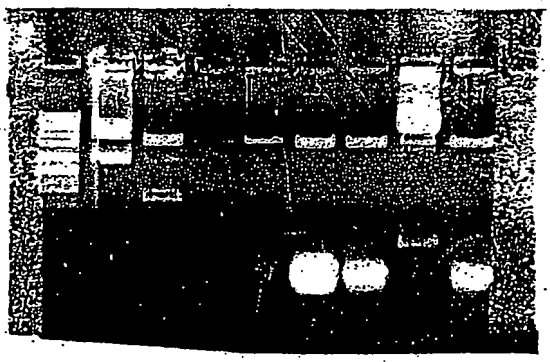
Cut 5 ml Ban 4

5 4637
10 10 x 3
82 DDO
3 Ba

(8) JKK Ban

Cut 10 ml Ban 4

10 JKK
5 10 x 3
82 DD
3 Ba



ISIS → PL ← ISIS

(9) ~~pBC 35 OCC 3' .35 SPE~~ pBS

~~Cut Swa~~ ~~Swa~~ Not/Apa

Not (3)

Other Apa

(10) pBC LT 22 .SCH

Cut Swa Apa/Swa Swa (4) Apa

Other Apa

(11) pBS OCC 3' .35 pro

Not/Swa Swa (4) Not

Other Not Other

(12) pBS 2×3's

Cut SMP Pst (3)

Then Heart

H3

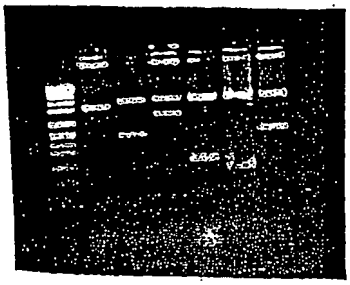
X

(13) pBC . SC4

HB (2)
Bkt
Not

X

(14) pBC . 35s pro Not/R1 Not/R1 (3)



9 10 11 12 13 14

(12) is no good ! Picked up a Act or H3 in one of the 3's
Must be in LT22! ??

Ligat	DS
Vector	1ul (9)
Insert	3ul (10) or ~~4ul~~ 5ul (11)
DDW	8
10 x buffer	2
Ligase	1

Descript → PL ←

Repeat the PUY:TMW ligation using vehicle back
2x vecta – half insert

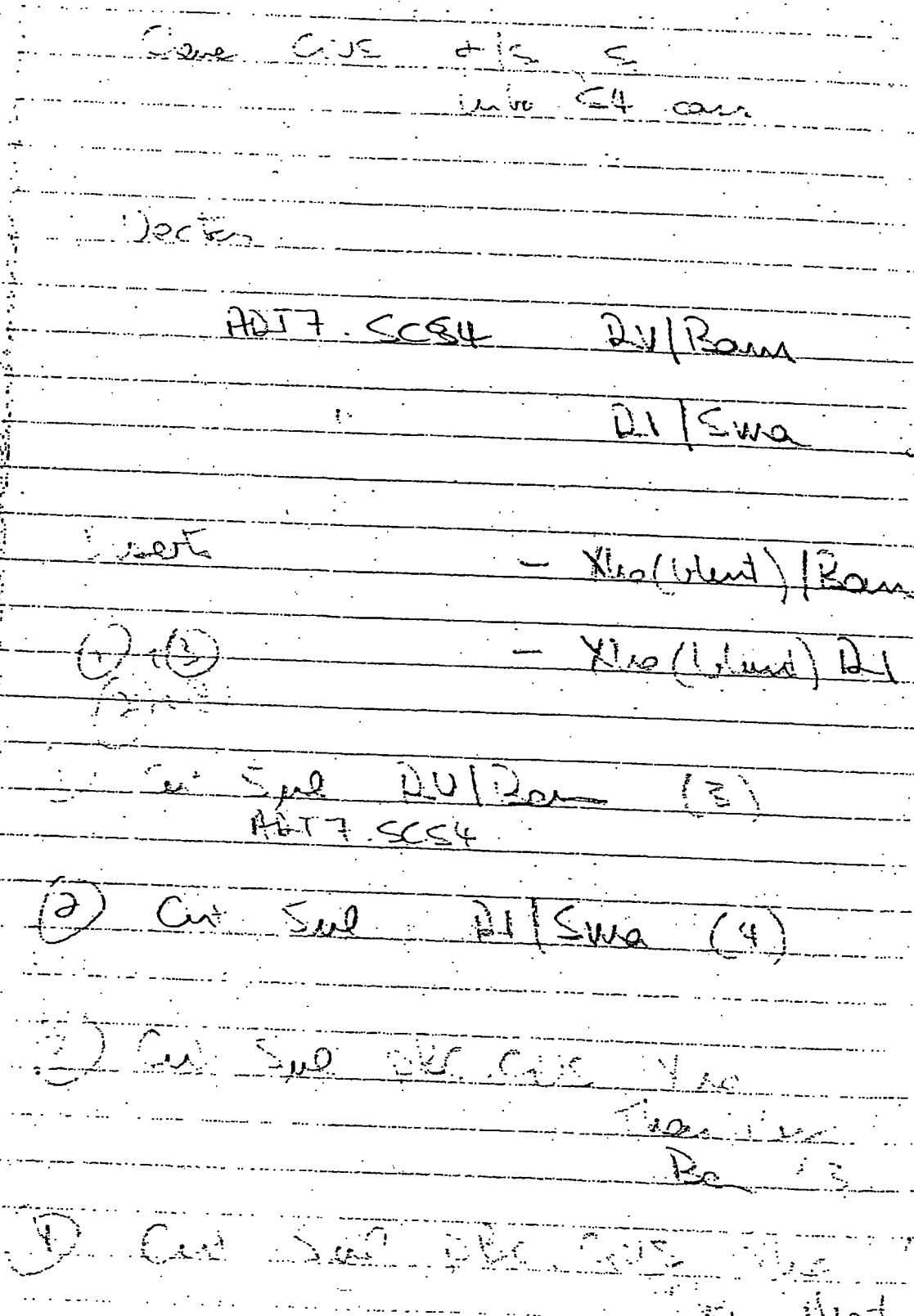
	X1	X2	X3	X4
Vecta	4ml (4)	2ml (5)	1ml (6)	4ml (8)
Insert	1ml (1)	5ml (3)	2ml (7)	2ml (7)
	1ml (2)	0.5ml (1)		
10x buffer	2	2	2	
DDW	11	9.5	14	11
Ligase	1	1	1	1

Descript. PUY TMW (1) JLK | JLK | JLK | JLK

Plate KBIW | KBIW | KBIW | KBIW

C/S Amp | Amp | Amp | Amp

Légendes



(5) pBC.GUS BamHI (3)

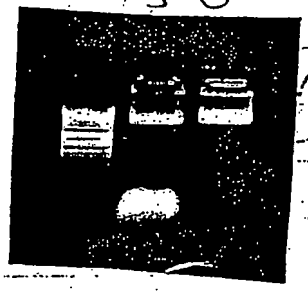
(6) 4637 BamHI (3)

Gels 1 2 3 4

(1) Something is wrong

(1) + (3) Leave it

(2) + (4)



	G1	G2
Vect	4µl 2µl (2)	2µl (5)
Insert	5µl (4)	2µl (6)
DDW	10	13
Ligase	1	1
10× buffer	2	2
Control	pBC SC54.GUS 2-sense	← TMW Ben pBC.GUS.

Plate	Amp	Cm
5/5	Cm	Amp

	G3	G4	G5
Veck	2nd (5)	2nd (7)	2nd (11)
Invert	2nd (6)	4th (8)	4th (9) 10th (10)
DDW	13	11	–
10 x bluff	2nd	2	2
Ligas	1st	1	1

Description: pRC GUS + TMW P2 | BUY R(H) JKK | 2 x pennants

Plate: Cu | Ka R(W) | Ka R(W)

Select: Mp | Mp2 | Cu

Transfer: - G2, - G3, - G4, - G5, - pRC GUS | LK | G4

Minis
- 1-3 Single Cu Mp5 G2
- 4-6 Pools of G2 Cu Mp5
- 7-9 Single Cu Mp5 G3
- 10-12 Pools of G3 Cu Mp5
- 13-15 Single white Ka Mp5
- 16+17 Pools of 4 G4
- 18 Single G5

Minis
- 1-3 / Single Cu Mp5 G2
- 1
- 10+11 Pools Ka Mp5 G4
- 12 1x G5
- 24 1x G5

1-3 Single

Cuts Mb
- 1-12 Ba (2)
- 13-17 Ba (H3) (2)
- 18 Ba (R1) (2)

168

Z1

1-12 ADT7 . CUS

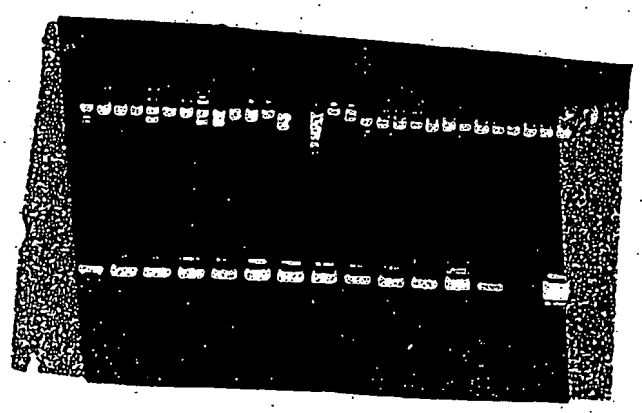
Z2 JLK . PUY . TMV

13-24

Z3 PBC . CUS . TMV

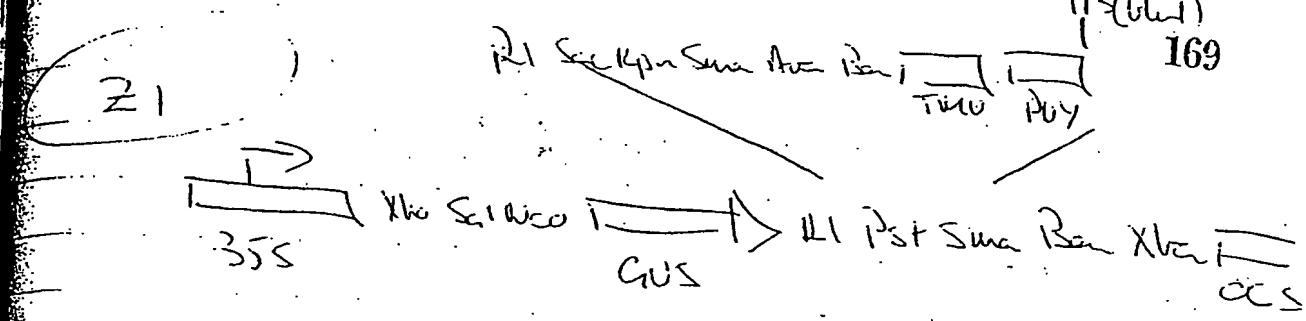
25-36

	Z1	Z2	Z3
Vector	2nd (8)	2nd (6)	5nd (4)
Invert	4nd (7)	4nd (5)	3nd (5)
DDW	11	11	9
Wxligne	2	2	2
Ligne	1	1	1
Mode	Amp	Ram	Cur
Select	Cur	Amp	Amp.

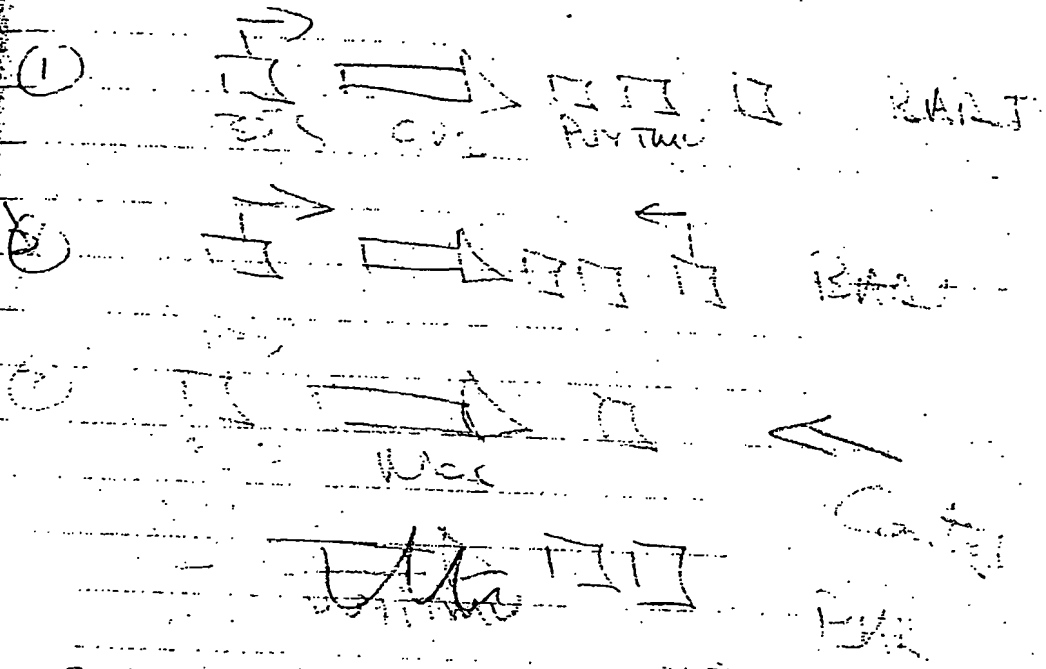


Z1

35 is probably ne good!



For manufacture of a clock



(1)

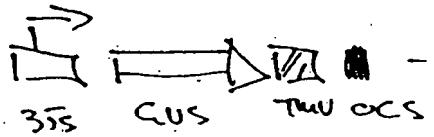
(2)

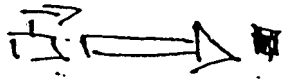
(3)

(3)

GUS.TMV for a GUSb plants.

Wat

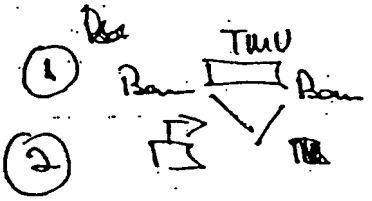
35S.GUS.TMV 

35S.GUS 

35S.TMV 

(4)

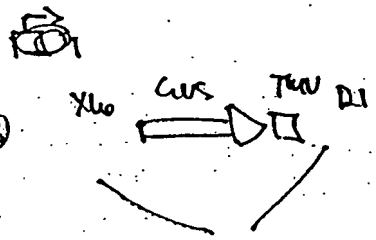
Have (Xho) GUS TMV (A1) pBC 23.35
pBC.GUS.TMV



4637 Ban

ALT 7 Ban

(9)
(10)



← Do 35+35

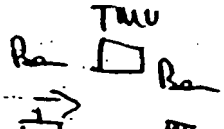
23.35 Xho | ~~Kpn~~ Not (blunt)

Sma
Ban ALT 7 Xho | ~~Kpn~~ ~~Not (blunt)~~
-Xho A1

(1) 10 µl 4637 Ban (3)
(2) 10 µl ALT 7 Ban (3)
(3)a 10 µl 23-33 ~~Kpn~~ Not then blunt then Xho (3)
(3)b 10 µl -35 Not then blunt then Xho (3)
(4) 10 µl ALT 7 Sma then Xho (4)
(5) 10 µl pBC.GUS.TMV Ban (23-33) (3)
(7) TKK TMV Ban
(8) ALT 7 GUS Ban
23 # 4
(1) LA. ~~Kpn~~ TKK. DIIX D. 1128-21 12D

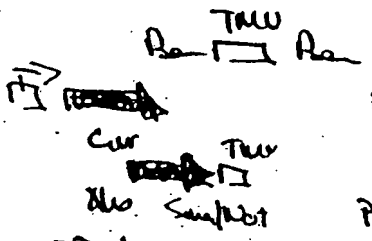
4 Ligatics

A1 ADJ7.TWV



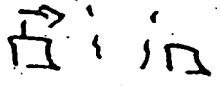
pRC.CUS.TWV
⊄ Ba (5)
ADJ7 Ba (2)

A2 ADJ7.CUS.TWV
#1

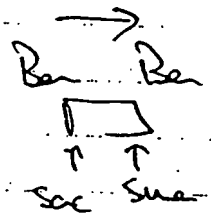


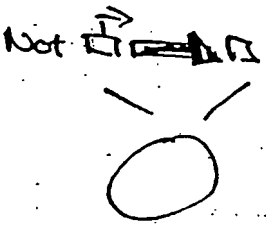
pBC.CUS.TWV (
ADJ7.CUS
Ba
pBC.CUS.TWV (
ADJ7 Xlio/Cu

A3 ADJ7.CUS.TWV
#2



A4 BADJ.CUS



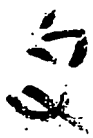


(10

(9

	A1	A2	A3	A4
Vector	2ul (2)	2ul (8)	3ul (4)	3ul (9)
Insert	4ul (5)	4ul (5)	5ul (3a)	4ul (10)
DDW	11	11	9	10
Window	2	2	2	2
Ligase	1	1	1	1

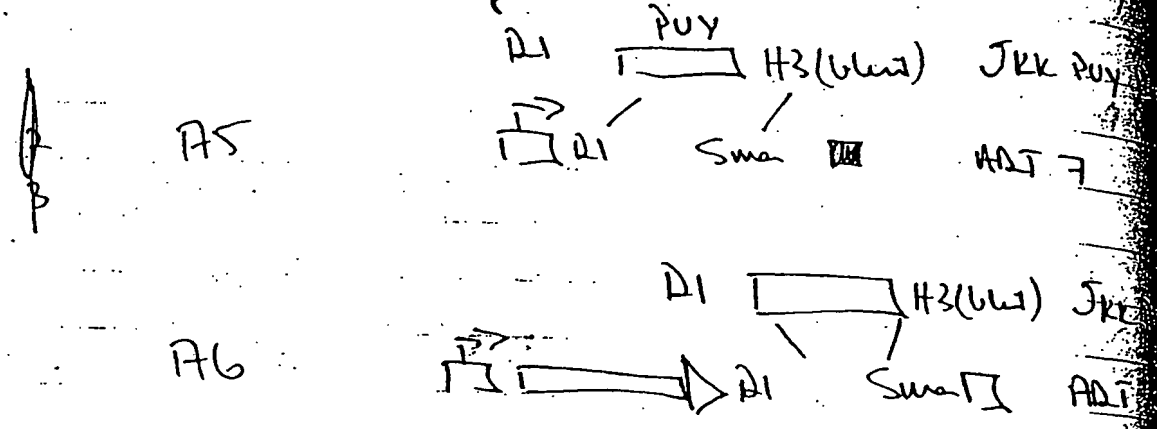
Description	ADJ7.TWV	ADJ7.CUS.TWV #1	ADJ7.CUS.TWV #2	BADJ.CUS
Plate	Amp	Amp	Amp	RW Strep
Counterselect	Cm	Cm	Cm	Amp



1-12 A1
13-30 A2
31-36 A4

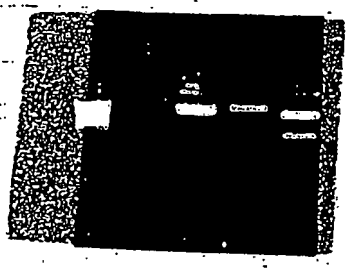
Cut
Not I
Not IR1
Not I

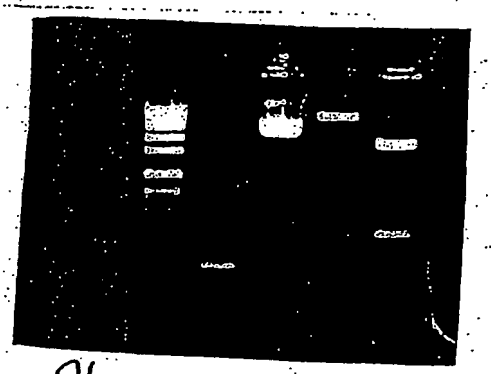
2 Ligation



DNA

1 ADT 7 R1/SmaI
2 ADT 7. CUS R1/SmaI
3 JKK PUY BamI/H3 R1/H3 (blunt)





	A5	A6
Vector	1ul 1	2ul 2
Insert	4ul 3	4ul 3
DDW	12	11
10xbuffer	2	2
Ligase	1	1
Descript	ADT7-PUY	

Plate
Select

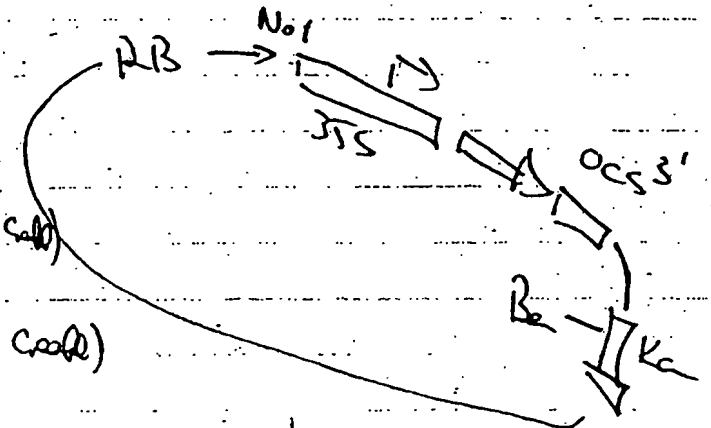
Shot Thursday (23) (29) (30) LP x 8 LP$\bar{o}\bar{e}$ x 2

Tuesday (16) (19)

3 x Ps

~~BART. GUS~~

Orientation of all liveries is ...



BART. TMU Light #1 (p31 Gradd)

BART. GUS (#33 p30 Gradd)

BART. GUS. TMU Light 2 # 5 (p31 Gradd)

9/9/94

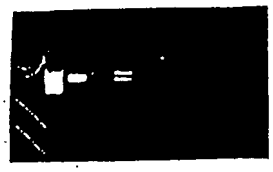
Skin 16 3 pieces

(19) 3 pieces
+ 2 x 3TS
+ 2 x 180 -ve catel

Cut

(11) iGUS SmallRL (4)

(12) PBC BU/RL (3)



(13) JKK PUY H3Kcal Bar (3)

(14) MGOO2 Bar (3)

10 OK
11 partial!
12 didn't cut
13 no DWH
14 didn't cut

Degrew

Degrew

PUSH THROUGH

Geoff sampled

(1) (3)

(2)

This is the way it's on the sheet

MMWA BO (1) (2)

(3)

Further cloning

DP2

Intermediate

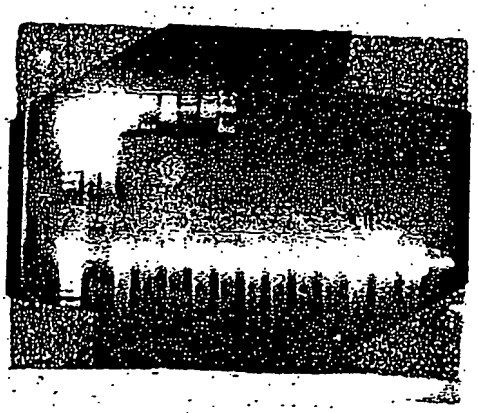
1. pBC.SC4 Not/R1 Cut5ml buff
2. pBC. 355 Not/R1
3. JKK R1

4. pBC.5503 1-5
   Pst 3 ✓
   Blunt
   HindIII

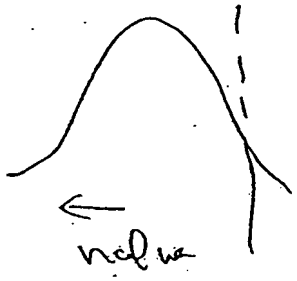
5. ADT.SC4 Bam 3 ✓
   Blunt
   HindIII

6. HDT7 Bam/Xba 2 ✓
   blunt

7. pBC.SC4 ~~Not/H3~~ Not (3) ✓ 2^00 6+7
   ~~blunt~~ H3 (2)
   Blunt



Nb



Ce

Monday

Cresol + Tobacco TF

- B.S.
- Petra S1NC
  S7NC
- M.C. BART. TMV

Tuesday

4 x a CWSb Lines

Neil

- BART. CWS
- BART *

} 2 plates each

Wednesday

Neil Dilution series into W38

SHOOT IMC. Switch on ?

(1) — small box

Tighten nut bracket

(2) Cold side (1) Grid

(3) Screw on top

Upside down

(3) R1/Pst (6) pBC. 2×3's (3) ✓

(4) R1/Pst (9) pBC. 2 pauses (3) ✓

(5) McCOS GEM3Z. PUX. TMW. (4)

~~Pst~~ ~~Sma~~ Kpn BlnI (10)

(6) pBC. CUS (4)

Pst
Sma

pBS SSO 3'
~~Sma Bam Spe~~ Xl

1] Pst Sal Clo H3 R1
SSU

(7) McCOS

Bam (3)

(8) pBC 2×3's R2/R1

(9) pBC 2 pauses Sma/R1

(6) pBC.SK+ Not (3)
HindIII (2)
Blunt

(7) ADH7 Ban/Xho (2)
Blunt

Ligation

	6	8	12	
	X1	X2	X3	X4
Vecteur	2ul (1)	2ul (3)	2ul (5)	1ul (7)
Insert	–	2ul (2)	4ul (4)	6ul (6)
Oxbuffer	2ul	2	2	2
DDW	15	13	11	10
Ligase	1	1	1	1

Desc	2pas ADH1	pBC.SSO3' SCII	pBC.GUS PUV.TMV	JP3 (?)
Plate	Kan	Cm	Cm	Amp
C/select			Amp	Cm
		Cle		
Cut	Not 3	Sma ⁂ 2	Pst/RI 3	

Preparative cuts

X	10	pBC 2× 3's	DW/DW	3
X	11	MC006	Swan/RJ	4
	12	pBC.SC4 Bam		3
		blunt		
		Sal		3
	13	pBC.SSU3'		
		Pst		3
		blunt		
		Sal		3
	14	pBC.SC4	Not	3
		Not/Xba/BB	Hind III	2
		blunt	blunt	
	15	Aft 7 Bam/Xba		2
		blunt		Dee

Ligation

	X10	X11
Vector	2ml B	1ml 15
Insert	4ml 12	4ml 14
DDW	11	12
10×buffer	2	2
Ligase	1	1

Description

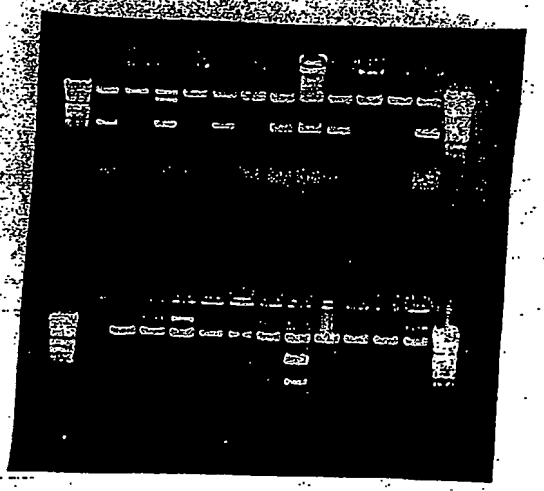
Plate	pBC.SSU3'	13 14
Select	Cm	Amp
	-	Cm

B7 (69) % #1 as PBC.GUS.PUY.TMV

B5

RTC 43 44

20
Grew % as DP3 ! hp



Rhizo 1-6 B9 Cla (1)
7-12 B8 Sma (4)

Rods 1-6

Rods 10 & 12 have B8 which is DP2

Here the following vectors

V1 ADH1 Sma / Xho (4) (I1)

V2 DP2 Hind III (2) (I1)
MG10 Blunt
Xho (2)

V3 DP2 Spe (1) (I2)
MG10 Blunt
Pst (3)

V4 DP3 Sal (3) (I2)
MG11 Blunt
Xho (2)

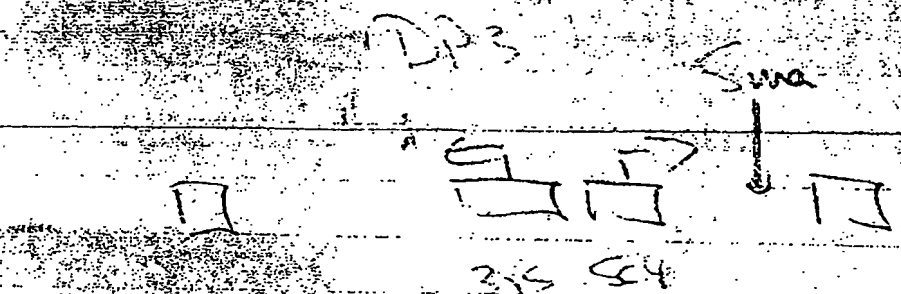
V5 DP3 Spe I (1) (I3)
MG14 Sma (4)

(1) pBC SC4 Not (3)
Blunt
Sal / Pvu2 (3)

(2) pBC SSU3' Pst (3)
Blunt
Sal (3)

(3) pBS SSU3'OCS3'
Pst (3)
Blunt
Sal (3)

(4) pBC.SC4
Not
Blunt
Sal I



both cons.

Sat/Rt blot

Sat

Bath cons

Lib

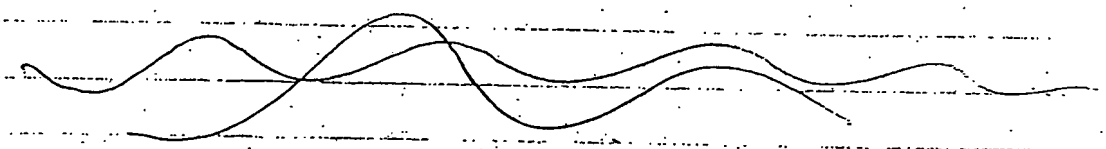
Sat

*Mapping DP3 Xho pBC SSU3'

Xba HindIII

pBC 2x3's } Xho

pBC SSU3' } Xba



VVI

Monday

X Cloning into DP1

UDP1 1 DP1 Xho (2)
(MCO16) H3

UDP1 2 Xho (2)
Bluni
HindIII (2)

UDP1 3 Xho (2)
Xba

UDP1 4 Xho (2)
EcoRV

IDP1 1 pBC.GUS.TMV.PUV (MCOOG)

Sal (3)
HindIII (2)

IDP1 2 MCOOG

EcoRI (2)
Bluni
HindIII (2)

IDP1 3 MCOOG
Xho (2)
Xba

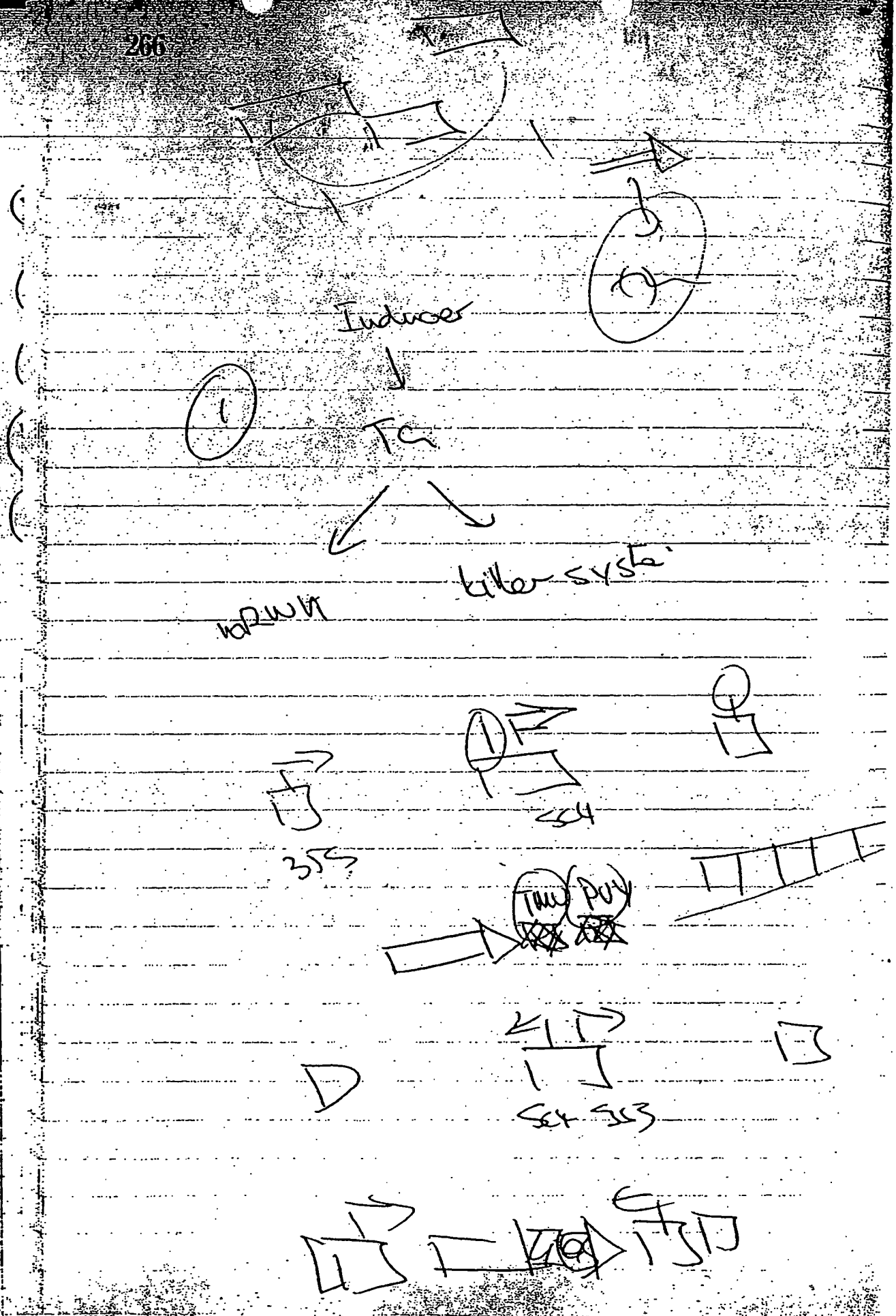

Inducer
TC
Killer system

EXHIBIT 2

PLANT INDUSTRY
CSIRO AUSTRALIA

CSIRO Division of Plant Industry
Institute of Plant Production and Processing

Postal Address
GPO Box 1600
CANBERRA ACT 2601
Australia

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Black Mountain, Canberra ACT
Tel (061) 246 4911 Int +61 6 246 4911
Fax (061) 245 5000 Int +61 6 246 5000

FACSIMILE TRANSMISSION

John Slattery
Davies Collison Cave
GPO Box 4387QQ
Melbourne VIC 3001

Dear John,

Re: **Patentability of new approach to gene inactivation**

Attached is an outline of an idea that Mick Graham has regarding antisense technologies. We would like to get your opinion as to whether we have enough basis to file immediately for a provisional patent.

The concept emerges from the fact that introduced genes (transgenes) are capable of suppressing expression of endogenous genes when the transgenes are in either the normal or reverse orientation. The reverse orientation approach has become known as antisense; the normal orientation is becoming known as sense co-supression. Both antisense and co-suppression are the subject of existing patents.

It has been thought that antisense works by the binding with the opposite endogenous mRNA sequence thereby preventing translation of the message, but there has been no definite proof that this is the method that operates *in vivo*. On its own, such a mechanism cannot account for the co-supression that occurs when a transgene is inserted in the sense orientation.

Mick's hypothesis is that, in some instances, sense transgenes become inserted in chromosomal positions where a partial (or even complete) antisense transcript is produced, thus leading to a similar sense/antisense mRNA hybrid molecule forming. Further it is suggested that it is not the passive formation of the mRNA hybrid alone that prevents translation (expression), but rather this hybrid induces an endogenous mechanism that destroys such hybrid RNA molecules in a sequence specific way (perhaps the action of a ribonuclease?). If this proves to be the case it leads to a number of new ways of exploiting this phenomenon, some of which are outlined in the attachment.

A range of scientific evidence now seems to be pointing strongly in the direction of this theory and Mick feels that it won't take long for other research groups to come to similar conclusions (if they haven't already done so). Thus there is a strong sense of urgency about getting as early a priority date as possible. It should be possible to prove or disprove the hypothesis within the 12 month period. We should be able to produce the transgene constructs outlined in the attachment and determine whether they have the postulated gene suppression effects in transgenic organisms.

My view is that this idea synthesises existing and emerging knowledge of both "antisense" and "sense" suppression of gene expression into a novel hypothesis as to the fundamental mechanism leading to these effects. If this proves to be the mechanism operating in such transgenics, then it leads to a range of novel

...oaches to down-regulation of gene expression that would appear to be advances on (or at least ...enting around") standard "antisense" and "sense" approaches. Is this inventive enough (if proven by subsequent work) to justify a patent application, and should we go ahead with a speculative provisional based on the idea because of the potential value of this technology?

I would appreciate receiving your opinion on this as soon as possible so that we can proceed to prepare more detailed information for a provisional patent application if it is warranted.

Sincerely,

Allan Green
Principal Research Scientist

cc Mick Graham
T.J. Higgins
Pat Walsh
Jim Peacock

Rationale

We consider the mechanism of "anti-sense" and "co-suppression" are identical. Both involve a change in the function of the transgene, from producing a normal mRNA to interacting with endogenous factors, which results in the transgene mRNA functioning as a sequence specific RNAase.

This "state switch" requires an interaction between the transgene and the endogenous homologue - this interaction probably involves hybrid formation between the endogenous mRNA and the transgene.

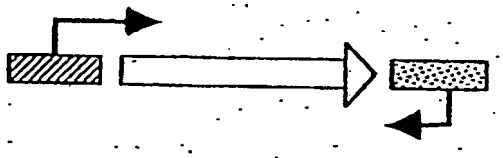
The difference between a weak and strong phenotypic effect in different transgenic lines results from differences in the propensity of the sense or anti-sense transgene to switch states. The differences in sensitivity is possibly a consequence of transgene integration into existing transcriptional units.

Improving existing strategies to create strong phenotypes

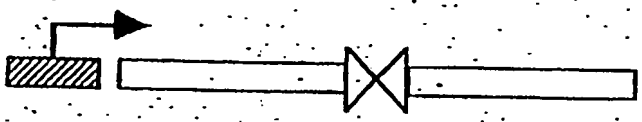
In order to obtain consistently strong phenotypic effects, constructs with a stronger propensity to switch states can be designed. These will produce RNAs which will form double stranded hybrids thereby resulting in an increased likelihood of inducing the state switch.

Possible constructs envisaged are shown below. The shaded boxes with the filled arrowheads represent promoters, the arrow shows the direction of transcription. The open boxes with open arrowheads represent coding sequences, the arrow representing the sense orientation of the gene.

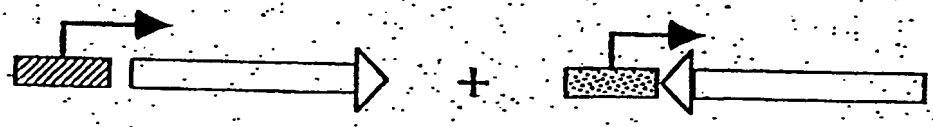
Case 1: Two promoters drive expression of the same transgene in opposite directions resulting in formation of complementary RNAs that can form hybrids, hence switching states.



Case 2: A single promoter drives expression of an inverted repeat. The transcript can then form a hairpin hybrid hence switching states.



Case 3: A sense and anti-sense construct are introduced into the same cell either as a consequence of a sexual cross or by super-transformation. The two seperate transcripts can then form a hybrid hence switching states.

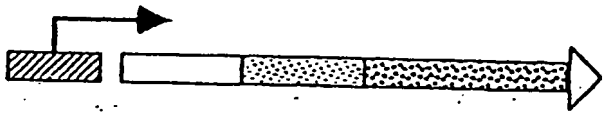


Another implication of this model is that the use of hybrid RNAs will permit the design of constructs that will inactivate multiple genes. Once a gene switches state the sequence specific RNAase activity will be a property of the complete RNA sequences of the switched transgene. Thus any endogenous gene that contains sequences homologous to sequences in a switched hybrid transgene will be suppressed.

Such constructs might be used to induce the state switch more rapidly using a more abundant or constutively expressed RNA as the endogenous inducer.

In addition single constructs consisting of a promoter driving hybrid sequences should inactivate expression of all endogenous genes present in the hybrid RNA. This might for example permit the design of single gene constructs to protect against multiple viruses.

Case 4: Different portions of the hybrid RNA are shown by different shading. A single promoter driving this construct (or constructs designed as for cases 1, 2 or 3) should supress the expression of all endogenous genes whose sequences are present in the hybrid. The state switch can be induced by interaction with only one sequence, but the consequence will be supression of all sequences present in the switched transgene.



Expected outcomes

- These strategies should permit the design of constructs that will be more effective at creating transgenic lines with strong suppressed phenotype. For example, typically 1 in 10 transgenic lines for a given construct will display a strong phenotype. The use of constructs with a higher propensity to switch should result in a higher proportion of transgenic lines showing strong phenotype.

- Intermediate phenotypes resulting from anti-sense or co-suppression are almost certainly phenotypically chimeric and intrinsically unstable. These constructs should result in a lower degree of such chimerism and an increase stability of phenotype.

- For case 4 novel strategies can be designed to inactivate multiple genes. For example hybrid RNAs consisting of sequences from different viruses should protect against all those viruses once the state switch occurs.

- Such constructs should be useful in mammalian systems.

EXHIBIT 3

PROVISIONAL PATENT SUBMISSION

Introduction

This invention describes a novel approach to creating gene "knockout" phenotypes in plants and animals by transgenesis. The invention will permit the efficient creation of plants and animals with little or no expression of the targetted gene or genes, including the creation of viral resistant or immune plants or animals.

This is currently achieved using anti-sense or co-supression. Both methodologies seem to act by the same mechanism, namely activation of an endogenous sequence specific RNA degradative system. Activation of this endogenous system usually requires a "trigger", namely appearance of the target mRNA or viral RNA in cells of the organism.

This invention describes the design of transgenes which are either more sensitive to the trigger, or trigger themselves. Engineering plants and animals with these constructs will result in:

• A higher proportion of transgenic organisms expressing strong "gene knockout" phenotypes. Only a low proportion of transgenics produced using anti-sense or co-supression display strong phenotypes, most display sectoring, namely areas of tissues or organs are viral immune or do not express the target gene, whilst adjacent areas express target genes normally or support normal levels of viral replication.

• More stringent "gene knockout" phenotypes. For constructs with more sensitive triggers, activation of the RNA degradative system will therefore occur earlier in development with less inducer. Thus with viruses for example little if any replication will occur before immunity is induced, whilst for "gene knockouts" little if any expression of the target gene will occur before the system is induced. For constructs that trigger themselves no inducer will be required, resulting in true viral immunity or "gene knockouts".

• Phenotypes will be more stable. Both anti-sense and co-supressed phenotypes are unstable, the phenotype can revert during development resulting in complete reversion or sectoring.

• Strong phenotypes will be obtained in heterozygotes or proimary transgenics.

CONCEPT

1. The invention is based on recent observations indicating that anti-sense and co-supression function by activating an endogenous RNA degradative system.

2. There is a trigger which activites of the endogenous RNA degradative system. The triggering event is usually induced by the presence of the target RNA, either the virus or the targetted RNA.

3. Rare transgenic insertions trigger themselves, presumably through interactions between repeated inserts.

4. Novel transgene design can permit:

- Transgenes with an increased sensitivity to trigger early in development.
- Transgenes that trigger themselves.

5.

DEMONSTRATION

1. Tobacco were transformed with the constructs shown in Figure 1:

A. GUS PVY/TMV fusion driven by the 35S promoter

B. An anti-sense GUS PVY/TMV fusion driven by the SCSV 4 promoter.

C. A "double promoter construct" (DP2): containing two transcriptional units, a PVY/TMV fusion driven by the 35S promoter and an anti-sense GUS PVY/TMV fusion driven by the SCSV 4 promoter.

D. A "double promoter construct" (DP1): containing two opposing promoters driving expression of the GUS PVY/TMV fusion.

2. Primary transformants were infected with PVY using manual innoculation.

3. Symptom development was monitored visually, and PVY replication was monitored using an ELISA assay

4. Results indicate that a much higher proportion of plants transformed with construct C were resistant to PVY (Table 1).

EXHIBIT 4

MAPPING TRANSGENE ACTIVITY IN PLANTS USING VISIBLE PHENOTYPES

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Introduction

Transgenic plants are relatively easy to create. Typically to analyse transgene expression many primary lines are generated but only a few showing the desired expression pattern or phenotype are selected for more detailed analysis; lines showing unanticipated expression patterns are frequently discarded. Much valuable information regarding transgene expression is lost during this culling process, in particular evidence refuting tacit assumptions that transgenes normally behave in a uniform, predictable Mendellian fashion.

Transgene expression is usually monitored using biochemical or molecular assays. However the information gained from such approaches can be of limited value, especially when spatially or temporally regulated processes are considered. For this reason visual marker genes such as GUS or ß-galactosidase are frequently employed. Marker genes can provide more detailed information on gene expression and since assays are quick and simple, large populations of transgenic lines can be more readily analysed.

In plants, transgene expression itself sometimes creates visible phenotypes. Figure 1 shows an example of a single leaf from a transgenic *Flaveria bidentis*, a tropical C4 plant. This plant was transformed with a construct designed to inhibit expression of the key photosynthetic enzyme pyruvate phosphate dikinase (PPDK). In green tissues of this leaf both PPDK activity and photosynthesis are normal. In contrast PPDK activity is not detectable in the yellow areas, which are consequently non-photosynthetic. If this whole leaf were ground up and assayed conventionally, PPDK protein levels, enzyme activities and mRNA levels would be about 50% compared to controls. If there were no visible phenotype these data would most likely be interpreted as reflecting a uniform reduction in gene expression across the entire leaf. Clearly in this example complete reliance on biochemical or molecular data would be misleading, and we suggest this precise situation frequently occurs when analysing transgenic organisms.

Based on our own results and a review of the literature we show similar visible or easily scored phenotypes are remarkably common in transgenic plants; however we believe until now neither their frequent occurrence or full significance has been appreciated. In these cases transgenes behave formally as visible marker genes, thereby providing simple assays which precisely define changes in gene expression. By examining such phenotypes large populations of transformed lines can be analysed, but more importantly changes in gene expression can often be directly visualised in space (position in a plant or tissue) and time (stage of plant growth or development).

Sectored phenotypes in transgenesis

Visible sectored phenotypes are quite common in transgenic plants. Examples from our own work are shown in Figure 2 and others from the literature [1-15] are listed in Table 1, and we are also aware of many further unpublished examples. Detailed descriptions of these plants and phenotypes can be found in the Figure legends and Table footnotes. Many of these phenotypes occur when an extra copy of a gene is expressed in either sense or antisense orientation and hence reflect sequence specific *trans* inactivation events targeting endogenous genes. However sectored phenotypes sometimes occur when heterologous sequences such as bacterial genes are expressed in transgenic plants.

Sectored phenotypes also occur in viral resistant transgenic plants [16-19], examples are shown in Figure 3, and others from the literature are listed in Table IL These viral resistant phenotypes manifest as alterations in the distribution of viral symptoms which for some viruses provide an indirect visual assay for viral replication (Fig. 3).

Not only are such phenotypes widespread, but often a high proportion of independently transformed lines show sectored phenotypes (Table 1). Frequencies range from about 10% to close to 100% of transgenic lines.

The Significance of Sectored Phenotypes

These phenotypes are significant because they occur in a variety of different circumstances, namely antisense, co-suppression and some instances of viral resistance and transgene instability. Such terms are used to describe phenomena that remain poorly understood at the molecular level, but they are commonly assumed to reflect distinct molecular processes.

In this context the occurrence of sectored symptoms in viral resistant plants is particularly significant. The molecular processes underlying RNA-mediated viral resistance were recently brought into sharp focus by Lindbo *et al.* [20], whose results define remarkable posttranscriptional processes responsible for resistance (for recent reviews on gene silencing in plants see refs. 20-26). Their data, which will be considered in more detail below, indicate viral resistance reflects sequence-specific degradation of viral RNAs. Formal evidence links viral resistance and at least some instances of transgene instability, indicating that mRNAs from nuclear genes can also be degraded by the same mechanism.

The sectored patterns of gene activity described above are only apparent because the events give visible or easily scored phenotypes. There is no reason to suspect that the majority of constructs, which give no obvious phenotype, will not behave in a similar fashion; however in such instances sectored gene expression could be easily missed, since quite detailed analysis would be required before it could be detected. We propose therefore that sectored gene activity occur much more frequently in transgenic organisms than previously imagined. Consideration of visible phenotypes in plants provides a simple means to visualise and more fully understand the full consequences of transgenesis.

Proposal: Sectored phenotypes arise from a common mechanism

We propose that phenotypic similarities of variable sectored patterns of gene activity, reflect common molecular events - namely activation of the posttranscriptional

homology-dependent RNA degradative system responsible for RNA-mediated viral resistance.

Initial evidence for this is based on overall phenotypic similarities that occur in transgenic plants. When considered together visible sectored phenotypes share several important characteristics. They are clearly non-uniform, regions of distinctly differing gene activity exist in sharply delineated sectors; these sectors often, perhaps always, correspond to regions of complete gene inactivation. Another striking feature is the extreme phenotypic variability that often occurs in transgenic lines; changes in gene expression occur unpredictably in space and time, generating remarkably complex phenotypes. These observations are consistent with activation of a simple binary switch which is variably induced in space and time, in what often seems an unpredictable fashion.

The available molecular evidence is consistent with this proposal and various other observations from the literature provide further indirect support. Remarkably similar events have been described in mammals and possibly other organisms, suggesting these observations have wide implications for understanding and interpreting transgenesis.

Sectored phenotypes reflect complete gene inactivation

For many of the phenotypes described above sectored regions seem to correspond to areas of complete gene inactivation, consistent with the complete degradation of mRNAs. Thus for *Flaveria* expressing PPDK sequences only background levels of enzyme activity are detected in yellow chlorotic sectors. Similarly, some phenotypes listed in Table 1, such as a complete lack of corolla pigmentation in petunia petals expressing CHS and DFR sequences or qualitative changes in starch composition in potato starch granules are also consistent with this notion. Most other phenotypes in Table 1 are fairly poorly described, but they are not inconsistent with this notion. Furthermore in instances of co-suppression where quantitative enzyme measurements have been reported, namely chitinase, ß-glucanase and nitrate reductase () complete inhibition of gene activity occurs. Mechanistically viral immunity reflects complete degradation of viral RNAs, sectors of complete gene inactivation would be anticipated if they arise by a similar process.

We are aware of some exceptions to these general observations which suggest two situations where apparently intermediate phenotypes might arise. In instances where a protein or its product are relatively stable one might gain the impression of intermediate levels of gene expression. This may be the case with NADP MDH in *Flaveria* (Figure 2), where sectored regions express about 5% of control enzyme activities. NADP MDH is a chloroplast enzyme and is therefore likely to be quite stable, complete inhibition of gene activity might only be observed long after gene inactivation events occur. Another exception might arise in instances where multi-gene families are targeted for *trans* inactivation. In transgenic *Gerbera hybrida* expressing antisense CHS sequences, some lines produce pink flowers from a red parent. This phenotype is consistent with partial reductions in CHS activity ( ), which could reflect either partial *trans* inactivation of CHS mRNAs, or alternatively result from complete inactivation of specific members of a multi-gene CHS family that might be expressed in *Gerbera* petals. A similar phenotype has been noted in a single transgenic petunia.

Unpredictable gene expression in transgenic plants

Transgene instability in the examples shown in Figures 2 and 3 results in highly variable, unpredictable phenotypes. These must reflect complex patterns of gene inactivation which occur frequently throughout development. In some instances evidence of cell lineage relationships can be inferred, whilst in other examples gene inactivation occurs in an apparently stochastic fashion.

For example, in *Flaveria* showing unstable PPDK expression each leaf on a plant shows unique patterns of gene inactivation (Fig. 2A,B). PPDK inactivation events must initiate differently for each leaf at different stages of development. The leaf in Fig. 2C illustrates this point. The lower half is nearly fully yellow, presumably a gene inactivation event occurred early in development and involved half the leaf meristem. In the upper half gene inactivation events probably occurred much later in development, and there have been many such events. This leaf also shows evidence of a reversion event, the large green sector in the bottom of the leaf. Moreover in such plants whole shoots can be fully green, whilst adjacent shoots become completely yellow (Fig. 2E and F). Sectoring in leaves from plants expressing NADP MDH sequences also results in extremely complex essentially random phenotypes (Fig 2 G-I) which presumably reflect cognate influences on gene inactivation.

Highly variable sectoring is also seen in mini-tubers expressing PPO constructs (Fig. 2 J-L). Some tubers show only a few PPO-expressing sectors, whilst others show large areas of PPO-positive tissues. Positive sectors presumably arise from one or a few cells which either retained or regained normal levels of PPO activity during tuber formation, cones of cells presumably reflect subsequent cell division which lead to radial expansion of the tuber. Some tubers from this line showed apparently normal expression of PPO, further emphasising the unpredictable nature of phenotypes.

When alterations in viral symptoms are considered similar unpredictable phenotypic variability also occurs in PVY-resistant tobacco (Figure 4). In some lines lesion numbers on third systemically infected leaves were about 5% of controls, whilst in others numbers were 30 - 50% of controls. Lesions typically appeared to be distributed in essentially random fashion, although some degenerate patterns were observed, for example different halves of a leaf formed distinctly different numbers of lesions. In one line a highly symmetrical pattern was observed, symptoms were confined to sharply delineated rectangular regions within interveinal tissues, similar to those described by Dougherty *et al* (1994). These phenotypes also showed marked developmental influences, lesion numbers usually decreased significantly in older leaves, but even this character was not invariant, in one resistant line the number of lesions actually increased.

The pigmentation patterns described in petunia petals expressing either sense or antisense CHS sequences are also remarkably variable. Different patterns occur in individual lines ranging from highly symmetrical to apparently chaotic. Most other examples of sectored phenotypes remain fairly poorly characterised, and have been variously described as "mottled", "chaotic" or "randomly distributed". We suggest careful consideration of these phenotypes will reveal much more information regarding phenotypic variability.

Transgene instability: current paradigms

To create transgenic plants DNA is most commonly introduced into regenerable tissues using either *Agrobacterium* or biolistics. Both approaches result in quasi-random integration of constructs. Thus in some lines constructs integrate as single copies, but often more complex patterns, such as multiple linked or unlinked integrations occur. Each individual in a population of transgenic lines therefore possesses a unique pattern of transgene integration and it is becoming increasingly clear that these markedly influence transgene activity, complex transgene integration patterns are frequently associated with aberrant expression patterns.

Position effects; cis *inactivation of gene expression*

The term "position effects" was coined to describe alterations in transgene activity that might reflect localised *cis* influences on gene expression. For example position effect verigation in *Drosophila* is thought to arise through localised influences of heterochromatin on gene expression, endogenous genes near blocks of heterochromatin, or transgenes which integrate near such regions, show abnormal verigated expression patterns. Similarly chance integration near strong enhacers, might influence either the total activity or developmental specificity of a particular promoter in individual lines. Similarly methylation of transgene sequences, which arises *de novo* following integration, can markedly influence gene expression, which is often thought to reflect promoter methylation leading to transcriptional silencing.

Trans *inactivation of gene expression*

At least two processes are known which can influence transgene expression in *trans*. Methylation patterns from transcriptionally inactive transgenes can be transferred to homologous sequences elsewhere in the genome, presumably through some type of somatic interactions between repeated sequences. Remarkably this can result in the transcriptional inactivation of unlinked locci. Although such processes have only been demonstrated for repeated transgene promoter sequences, it seems possible similar interactions could occur between duplicated coding sequences.

The second process is frequently referred to as co-suppression or posttranscriptional gene inactivation. The term co-suppression was originally coined to describe phenomena observed in transgenic petunia where attempts to overexpress key genes controlling pigment biosynthesis unexpectedly resulted in a complete block in pigment production in sectors of petals ( ). Expression of both the transgene and endogenous homologue were blocked in such lines, hence the term.

An extremely important shift in understanding co-suppression occurred recently with the demonstration that many instances of genetically engineered viral resistance in plants occur by this mechanism. Constructs designed to express viral structural genes in plants, such as coat protein ( ) or polymerase genes ( ), often confer viral resistance. This was originally thought to result from the inappropriate expression of viral proteins which were assumed to act through poorly defined *trans* dominant effects on viral replication ( ). However Lindbo et al (1993) showed conclusively that the expression of non-coding viral RNAs can confer strong viral resistance, the term RNA-mediated viral resistance has been coined to describe such resistance. Such viral resistant lines show varying degrees of resistance, which manifests as either viral immunity or "recovery" from viral infection - as plants grow new tissues become viral immune; the viral resistance phenotypes shown in Figure 3 are examples of this.

Northern analysis indicated this viral immune state was associated with markedly decreased steady state transgene mRNA levels, but nuclear run-on experiments showed transcription rates remained essentially equivalent to fully susceptible tissue. These same molecular changes, namely high transcription rates associated with low steady state mRNA levels, also occur in co-suppression of nuclear genes. These results indicated that both the transgene and viral RNAs are degraded in viral resistant lines through some previously unimagined posttranscriptional process. Such viral resistance is sequence-specific, since unrelated viral RNAs are not degraded, moreover RNA degradation must occur in the cytoplasm as potyviruses, which were targeted in these experiments, replicate exclusively in this compartment.

Such data indicated that both RNA-mediated viral resistance and co-suppression are posttranscriptional phenomenon explicable only in terms of activation of an endogenous homology-based RNA degradative system. In co-suppression this RNA degradative system targets mRNAs from nuclear genes, whilst for viral resistance viral RNAs are targeted.

Antisense

In transgenic plants antisense approaches are frequently used to specifically *trans* inactivate expression of endogenous genes. The mechanism of inactivation remains unknown but antisense is widely, although not universally ( ), thought to differ from co-suppression. Antisense phenotypes are commonly thought to reflect duplex formation between the antisense transgene and endogenous sense mRNA, which is believed to inhibit either translation of the targeted mRNA or mark it for destruction by unknown process(es). Such models imply that antisense represses gene expression uniformly in a whole plant or tissue, which seems a widely held assumption.

Sectored phenotypes reflect posttranscriptional gene inactivation

Many of the sectored phenotypes described above reflect sequence-specific *trans* inactivation events. Processes which act only in *cis*, such as position effect verigation, cannot be responsible for these phenotypes. Moreover whilst transgene methylation can inactivate gene expression in *trans* such processes cannot possibly account for viral resistance since most plant viruses have RNA genomes.

Support for this view is based on specific molecular criteria. In instances of posttranscriptional gene inactivation, nuclear run-ons and Northern blots show that inactivated genes are transcribed at normal rates but steady state RNA levels are low ( ). This contrasts to transcriptional gene inactivation where run-ons show genes are not transcribed.

A review of the literature shows that at least three instances described as co-suppression ( ) and three examples of RNA-mediated viral resistance ( ) satisfy these molecular criteria. We are not aware of any exceptions to this, and it seems reasonable to assume that most, probably all examples of these phenomena will occur by activation of this same RNA degradative system.

Furthermore at least two instances described as transgene instability have been reported where run-ons are also consistent with posttranscriptional gene inactivation. Whilst

visible phenotypes were not reported in these examples, these results indicate transgenes can inactivate their own expression through posttranscriptional processes. Transgene instabilities that might seem to occur in *cis* sometimes reflect *trans*-acting processes. It seems reasonable to assume other examples labelled unstable expression will also occur in this fashion, especially in instances where sectored phenotypes occur.

Less molecular data is available for antisense, we are aware of only one instance where results of nuclear run-ons have been reported. In tomatoes expressing antisense polygalacturonidase (PG) constructs, run-ons showed that transcription rates of both endogenous and antisense PG genes remain unaltered in ripening tomato fruit whilst steady state mRNA levels for both genes decreased markedly (). These results were originally interpreted using conventional models - duplexes were assumed to form between antisense and sense leading to the specific destruction of both RNAs. This interpretation is tautological, the data are equally consistent with degradation via posttranscriptional gene inactivation. We believe the latter explanation is most likely, since additional indirect evidence discussed below provides further support for this view.

Implications

Our observations suggest that posttranscriptional gene inactivation occurs very frequently in transgenic plants, which has wide implications for understanding and interpreting transgenesis.

Transgene Instability

Our observations indicate that the frequency of transgene instability has probably been grossly underestimated. This has disturbing implications.

Sectored gene inactivation events might prove difficult to detect in instances where no phenotype occurs, especially when phenotypic variability is taken into account. Fine scale sectoring in whole tissues, or gene inactivation events in parts of plants or in transgenic progeny (either sexual or clonal) might easily be missed without detailed analysis. Furthermore environmental influences can markedly effect phenotypes. For example the frequency of co-suppression in tobacco over expressing copropophyrinogen oxidase constructs varied markedly when plants were grown in different glasshouses, similarly plants that stably expressed a herbicide resistance gene in glasshouses showed sectored expression in the field.

Important determinants of transgene stability can be recognised from the available literature. Complex multi-copy transgene integrations have been correlated with both posttranscriptional and transcriptional gene inactivation. Single copy transgene inserts are therefore likely to express more predictably. Furthermore to circumvent *trans* inactivation events constructs should utilise heterologous promoter and coding sequences whenever possible.

The mechanism and consequence of antisense

We have shown above that sectored phenotypes often occur as a consequence of antisense expression. Such observations refute assumptions that antisense phenotypes

are uniform and suggests that experiments using antisense may have been frequently misinterpreted.

One prediction is that phenotypes generated by antisense and co-suppression should be identical - however for such a comparison identity must be considered in the context of phenotypic variability. In transgenic *Flaveria* expressing either sense (Figure 2H) or antisense (Figure 2I) NADP MDH constructs, very similar sectored phenotypes occur. Similarly in PVY-resistant tobacco expression of viral sequences in either sense (Figure 4C) or antisense (Figure 4D) orientation results in the same recovery phenotype. Data summarised in Table 1 provides further support for this view, at least three other examples, CHS in petunia, ankyrin in *Arabidopsis* and probably GBSS in potato, give similar phenotypes for antisense and co-suppression.

Such a view also provides a simple explanation for the poor correlation between antisense RNA levels and phenotype which has been often noted. As described above low steady state transgene mRNA levels reflect activation of the posttranscriptional RNA degraditive system. Phenotypes reflecting strong gene inactivation should therefore correlate with low transgene mRNA levels. However since phenotypes are sectored steady state mRNA levels would be expected to vary considerably depending on the tissue or developmental stage assayed. Significantly, poor correlations between transgene mRNA levels and viral resistant phenotypes have also been frequently observed.

One difference between antisense and co-suppression is the frequency of gene inactivation events. The available data are also summarised in Table 1. In one instance (ankyrin in *Arabidopsis* and CHS in petunia) frequencies are identical, but in other examples co-suppression frequencies are higher (eg PPO in potato), markedly so for PVY resistance. The reasons for such discrepancies remain uncertain but could reflect differing propensities for activating the RNA degradative system.

The term antisense is loosely used to describe a variety of phenomena. For example in transient assays small decreases in gene expression occur when large excesses of antisense are introduced into cells. This differs from the complete gene inactivation described above; perhaps there is an effect of duplex formation, but it is small. Clearly notions of what is precisely meant by antisense require re-evaluation.

Engineering Viral Resistance

Our observations suggest the mechanism of viral resistance is sectored viral immunity. Sectored symptoms have been described by others in viral resistant transgenic plants (Table 2), including viruses from widely divergent genera namely potyviruses, potexviruses and tospoviruses, indicating this is a widespread phenomena. For many virus plant combinations there are particular difficulties associated with interpreting viral resistance phenotypes which might obscure similar sectored symptoms. Thus some viral species like cucumber mosaic virus (CMV) naturally grow out of viral infections, whilst in others such as potato leafroll virus (PLRV) symptoms provide only indirect indications of viral replication. In other examples visible symptoms do not occur at all. We suggest sectored viral immunity is probably widespread, but has often been missed.

Three common observations are frequently made for viral resistance phenotypes. Firstly when plants are challenged with virus a proportion do not develop symptoms. In those

that do symptom appearance is frequently delayed. Finally resistance often shows dose dependence, high innoculums of virus can overcome resistance. The simple model in Figure 5 can explain these observations.

There is surprisingly little evidence indicating that protein expression plays any role in conferring viral resistance, but this view has become dogma. Our observations provide a rational basis for considering viral resistance phenotypes which might help clarify such arguments.

Trans *inactivation of gene expression*

The ability to completely *trans*-inactivate expression of endogenous genes, or degrade viral RNAs is clearly of major practical and experimental importance. One surprising implication of our observations is that complete *trans* inactivation occurs very commonly in transgenic organisms; the available technologies of co-suppression and antisense are extremely effective.

What is required however are methods to better control gene inactivation in space and time. It seems likely that significant advances can be achieved in this respect.

Posttranscriptional gene inactivation in other taxa

It seems unlikely that a remarkable process like sequence-specific RNA degradation would be confined to plants. Many examples of distinctly non-uniform patterns of transgene expression have been observed in mammalian systems, and several key observations suggest at least some of these events occur through identical posttranscriptional processes.

In a transgenic mouse line expressing an antisense myelin basic protein (MBP) cDNA, marked decreases in both MBP mRNA and protein levels where observed. Localisation of MBP in neuronal tissues from these animals, using antibody probes, revealed MBP was distributed in a distinctly non-uniform fashion. This is consistent with sectored *trans* inactivation of endogenous MBP expression. Furthermore in at least two examples where mammalian cell lines were transformed with antisense sequences, a poor correlation between state levels of antisense RNAs and the level of gene inactivation was noted, reminiscent of the observed situation in plants. Sectored expression of transgenes has also been noted in transgenic mice and transformed mammalian cell lines.

Phenomena described as antisense, co-suppression or transgene instability have been described in fungal species. Moreover antisense strategies have been widely used in the study of *Dictyostelium*, and some evidence indicated that RNA degradative processes play an important role in programming differentiation processes. Such observations suggest that posttranscriptional gene inactivation is widespread.

The biological significance of posttranscriptional gene inactivation

The endogenous system responsible for homology-dependent posttranscriptional gene inactivation has remarkable properties; it seems likely such a system plays fundamentally important roles in biological systems. Others have proposed the system may normally play a role in viral resistance in plants or protect against the activity of

transposons. However the likelihood the system functions in other taxa suggests more general roles, for example one can easily envisage regulatory networks based on homology-dependent posttranscriptional processes.

In transgenic plants activation of this system frequently results in the creation of seemingly random or chaotic phenotypes, however some lines display remarkably symmetrical phenotypes. Such observations suggest that posttranscriptional processes might play an important role in pattern formation.

Our observations indicate posttranscriptional gene inactivation acts as a binary switch, rather than a "volume control" for gene expression. Intriguingly some models of enhancer action suggest they also function act as a binary switch by increasing the probability of transcription in individual cells rather than increasing transcription rates as generally believed. Assumptions that gene expression is normally fairly uniform in seemingly homogeneous tissues are perhaps overly simplistic, distinctly non-uniform patterns of gene expression may occur frequently.

EXHIBIT 5

[a] Construct used to create transgenic line. Plants were mostly transformed using *Agrobacterium*-based systems, except for some transgenic petunia lines which were regenerated from electroporated protoplasts. Most constructs used the constitutive 35S promoter to drive transgene expression, exceptions are for DFR, in some instances the native promoter was used and CHS, where sectored phenotypes occur with promoterless constructs.

[b] Visible or easily scored phenotype which reflects transgene activity.

[c] Antisense refers to situations where constructs were designed to express antisense RNA sequences; Co-suppression refers to situations where sense sequences were expressed, usually full length coding sequences aimed at overexpressing a gene; transgene instability refers to situations where genes are expressed which have no endogenous homologue, such as bacterial genes.

[d] Frequency refers to the frequency of primary (T_0) transgenic lines displaying sectored phenotypes.

[e] Transgenic *Flaveria* expressed full-length antisense *Flaveria* PPDK sequences driven by the 35S promoter.

[f] Transgenic *Flaveria* expressed full-length antisense *Flaveria* NADP MDH sequences or full length maize NADP MDH coding sequences designed o overexpress enzyme activity. Both constructs were driven by 35S.

[g] Potatoes (cv Lehmni Russet) were transformed to express antisense potato PPO sequences driven by 35S.

[h] Constructs designed to express either sense ( ) or antisense ( ) sequences driven by 35S were used to transform petunia. Sectored phenotypes also arise when promoterless constructs are used. The phenotypes of these flowers have been particularly well characterised and show remarkable diversity ( ). In some instances developmental changes in phenotype ( ), reminiscent of the viral recovery phenotype ( ) have been noted.

[i] *Arabidopsis* ankyrin encodes a *trans* regulatory protein which seems to play a central role in signalling chloroplast biogenesis. Transgenic expression of ankyrin cDNA sequences creates sectored regions of leaf chlorosis, consistent with absence of this differentiation signal. An identical phenotype occurs regardless of whether sense or antisense sequences are driven by 35S.

[j] Potato tubers produce two types of starch, highly branched amylose and linear amylopectin. The two types stain differently with iodine; amylose containing starch stains blue, whilst amylose-free starch stains red. For plants expressing antisense potato GBSS sequences driven by 35S whole tubers treated with iodine stain blue in some regions and red in others, reminiscent of the antisense PPO phenotype described above. In addition individual starch grains contain blue centres and red outer regions. Since starch grains grow outwards this phenotype provides a temporal record of gene

expression in individual tuber cells. Inactivation of endogenous GBSS expression seems to be triggered early in tuber development, before starch grains are fully developed but after GBSS expression is induced. Some grains show blue concentric circles, consistent with multiple gene inactivation and reactivation events occurring over time.

k Constructs expressing sense DFR sequences and intact DFR genomic sequences, driven by the native DFR promoter, show sectored regions of unpigmented tissues, similar to those described for CHS.

l Tobacco transformed to express tobacco nitrate reductase sequences driven by 35S show a sectored leaf necrosis. This visible phenotype has not been extensively described, but extensive biochemical data suggests this phenotype shows developmental alterations, reminiscent of viral recovery phenotype. Quantitative enzyme measurements indicate complete gene inactivation occurs in mature tissue.

m Tobacco were transformed with constructs designed to constitutively overexpress tobacco SAM synthetase using 356S. Two phenotypes resulted; overexpression leads to leaf necrosis, but this was unstable and reverted to normal phenotype. Qualitative ?. This phenotype also showed developmental influences.

n Tobacco were transformed to express copropophyrinogen oxidase sequences driven by 35S. Overexpression was associated with leaf necrosis, which were described as occurring in "chaotic patterns".

o Tobacco were transformed to express yeast invertase sequences driven by 35S. "Chaotic patterns of necrosis" were reported which correspond to regions where invertase was expressed. No invertase activity was detectable in normal tissues?

p *Arabidopsis* were transformed to express bacterial *RolB* sequences driven by 35S. Constitutive expression of *RolB* generates an auxin hyper-sensitive response, leading to the creation of a severely stunted phenotype. Occasionally normal shoots develop from these plants , analysis of gene expression in these shoots indicates *RolB* is not expressed, and nuclear run-ons indicate this inactivation is posttranscriptional. Mutants of these transgenic lines which showed a higher frequency of normal shoots have been isolated, providing formal evidence that posttranscriptional gene inactivation involves a host system.

q Transgenic tobacco resistant to the herbicide sulfonylurea were created using 35S to drive expression of the bacterial *csr1-1* gene. This confers herbicide resistance by.... In field trials of herbicide resistant plants, sectors of sensitive tissue were described as "mottled, sectored leaves and whole plants".

Table 2: Sectored symptoms in viral resistance.

Virus [a]	Resistance Constructs [b]	Reference
Potato virus Y (PVY) [c]	PVY NIa	This paper
Tamarillo mosaic potyvirus (TaMV) [c]	TaMV coat protein	This paper
Tobacco etch potyvirus (TEV) [d]	TEV coat protein	
Peanut Stripe potyvirus (PStV) [e]	PStV coat protein	
Potato virus X (PVX) [f]	PVX polymerase	
Tomato spotted wilt virus (ToSW) [g]	ToSW N protein	

[a] Refers to the viral species for which resistance was monitored. These include four potyviruses, PVY, TaMV, TEV and PStV; a potexvirus (PVX) and a tospovirus (ToSW).

[b] Viral resistance in these instances is RNA-mediated. All constructs were driven by the 35S promoter. For PVY a portion of the NIa protease gene was expressed; for TaMV .... For TEV a non-translatable coat protein sequence; for PStV translatable and non-translatable coat protein sequences; for PVX sequences derived from the replicase gene (these were translatable but resistant was subsequently shown to be RNA-mediated) for ToSW translatable sequences from the N replicase gene which confer resistance through RNA-mediated mechanism.

[c] Symptoms shown in Figure 2.

[d] Symptoms described as "distinctly localised in chlorotic interveinal regions".

[e] Symptoms described as "symptomatic and asymptomatic areas" where "virus was detected only in areas where symptoms were visually apparent".

[f] Symptoms described as "ameliorated symptoms, characterised by isolated chlorotic lesions rather than confluent mosaic".

[g] Symptoms described as ".....".

Figure Legends

Figure 1. Sectored gene expression in transgenic plants. A single leaf of a transgenic *Flaveria bidentis* showing sectored inactivation of endogenous PPDK gene is shown. This plant was transformed with a construct expressing antisense *Flaveria* PPDK sequences driven by the CaMV 35S promoter. In the green regions of the leaf PPDK activity is normal, whilst in the yellow levels measurable PPDK activity is only 2% of controls. In the yellow sectors expression of the endogenous PPDK gene has been *trans* inactivated and these regions are consequently non-photosynthetic. Fifteen lines were transformed with this construct, twelve were completely yellow and three gave sectored phenotypes, further examples of which are shown in Figure 2.

Figure 2. Highly variable phenotypes resulting from sectored gene inactivation in transgenic plants. (A-F) Sectored inactivation of PPDK expression ion *Flaveria*. (A,B) Single shoots from transgenic plants similar to those shown in Figure 1, note each leaf shows a unique pattern of PPDK inactivation. (C,D) Single leaves from such plants showing complex patterns of gene inactivation. Completely green (E) and yellow (F) shoots of *Flaveria* which grew on the same plant. (G-I) Transgenic *Flaveria* expressing NADP MDH sequences driven by the 35S promoter. The pale yellow sectors result from photosynthetic quenching which reflects the low (5%) levels of NADP MDH expressed in these sectors. NADP-MDH is expressed at about 50% of control values in green areas of leaves in these plants. (G) Plants were transformed with a construct designed to over express maize NADP MDH activity, only gene inactivation events were detected in such lines. Note each leaf shows a unique pattern of gene inactivation. (H) Single leaf from a plant expressing the sense construct in G. (I) Single leaf from a plant expressing a *Flaveria* NADP MDH antisense cDNA driven by the 35S promoter; the phenotype is indistinguishable from that in H. (J-L) Potato minitubers showing sectored inactivation of PPO expression. These plants (cv. Lehmni Russet) were transformed to express antisense sequences from potato PPO cDNAs driven by 35S. Tubers were cut and exposed to air overnight as a crude indicator of *in situ* PPO activity. (J) The tuber on the left in was grown from a non-transformed control and turns uniformly black, the tuber on the right was grown from a transformed line. (K) Closer view of the transgenic tuber in (J), note cones of black and white tissues reflecting changes in gene activity. (L) Four tubers from the same line, note each shows unique patterns of gene inactivation.

Figure 3. Sectored symptom formation in viral resistant plants. Viral infection in plants often results in the reproducible development of characteristic disease symptoms. For a particular combination of plant and virus, particular types of lesions (e.g. chlorotic or necrotic spots or lesions on leaves) occur reproducibly during development of the disease. For some viruses disease symptoms occur only in those tissues supporting viral replication, alterations in the distribution or appearance of such symptoms in transgenic viral resistant plants therefore provide indirect visual assays for transgene activity. (A) Non-transformed *Nicotiana tabaccum* (W38) infected with PVY, leaves which form following viral challenge are invariably systemically infected and develop characteristic chlorotic lesions. In our hands this phenotype is easily scored from the third to the seventh systemically infected leaf, where the lesions are distributed quite uniformly over the entire leaf. After this point leaves are smaller and symptoms less obvious, necessitating the use of ELISA assays to monitor viral replication. (B) Symptoms in transgenic tobacco expressing non-coding RNAs derived from a portion of the PVY NIa cistron driven by the 35S promoter. Note sectored distribution of lesions, viral particles

(detected by ELISA assays) are found only in symptomatic areas, no virus was detected in asymptomatic regions. (C,D) Systemically infected leaves from plants expressing sense (C) or antisense (D) NIa sequences relative to the PVY genome. Virus is detectable only in areas where single lesions or small foci of lesions are apparent, the same phenotype occurs regardless of orientation. These plants display the recovery phenotype first described by Linbo *et al* ( ). (E,F) Symptom development in *Nicotiana benthemiana* resistant to TaMV. etc

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Co-suppression in mammals

Background

Recent advances in plant molecular biology have given important insights into mechanisms of *trans*-inactivation of gene expression in transgenic plants. By expressing RNA sequences driven by strong constitutive promoters transgenic plants can be created where endogenous genes can be targeted for complete inactivation or rendered viral immune. The ability to create similar phenotypes in animals has enormous implications for medicine and agriculture. This programme aims to transfer these emerging principles of gene inactivation in plants to mammalian systems.

In plants the term co-suppression refers to the inactivation of gene expression which sometimes occurs when extra copies of endogenous genes are expressed in a sense orientation in transgenic plants (for recent reviews see Meyer, 1995; Schell, 1996). Important new insights into co-suppression form the basis of this proposal. It has been known for some time that viral resistance can be created in transgenic plants can be created by expressing viral RNA genes; this resistance was initially thought to result from the expression of viral proteins, however more recent evidence indicates the mechanism is identical to co-suppression (Linbo *et al*, 1993; English *et al*; 1996). Furthermore it is becoming increasingly clear that antisense occurs by this same mechanism (Dougherty and Parks, 1995: Graham *et al*, submitted for publication).

Mechanistically co-suppresion involves sequence-specific degradation of RNAs, either viral RNAs or mRNAs from nuclear genes. Following infection viral resistant transgenic plants "recover" from viral infection, recovered tissues become immune to further infection. In viral immune tissues Northern blots and nuclear run-on experiments show that transgene RNAs virtually disappear even though their transcription rates remain nearly equivalent to those in viral susceptible tissues (Linbo *et al*, 1993). Identical molecular changes, namely markedly decreased steady state mRNA levels but essentially unaltered transcription rates occur in co-suppression (Brusslan *et al*, 1993; van Blokland *et al*, 1994 and de Carvalho *et al*, 1995) and antisense (Sheehy *et al*, 1988) in plants. Since co-suppression results in the sequence-specific inactivation of RNAs, the mechanism of viral immunity must involve sequence-specific destruction of viral RNA (nearly all plant viruses possess RNA genomes). Moreover co-suppression must be a cytoplasmic phenomenon since viruses that replicate exclusively in this compartment can be targeted by this process. Furthermore this RNA degradation is sequence-specific since non-related viruses or nuclear genes are not degraded by the process. Such observations indicate the existence in plants of a previously unknown host system capable of quantitatively degrading RNAs in a sequence-specific manner.

One striking characteristic of co-suppression is that remarkably complex phenotypes arise in whole plants (M. Graham; submitted for publication). Co-suppression and viral resistance manifest as unstable sectored phenotypes, regions of essentially normal gene activity or viral susceptibility occur in some tissues, whilst compete gene inactivation or viral immunity occurs in adjacent cells. We believe such unanticipated behaviour has lead to much confusion about the behaviour of co-suppression and as described places important constraints on experimental design.

Whilst co-suppression has not been formally demonstrated to occur in mammals several key observations suggest its existence. In at least three examples where mammalian cell lines were genes have been inactivated using antisense approaches, a poor correlation between steady state levels of antisense RNAs and the level of gene inactivation was noted (Moroni *et al*, 1992; Kook *et al*, 1994 and Thomson *et al*, 1995). This is reminiscent of the degradation of transgene RNAs associated with co-suppression and antisense in plants. In a transgenic mouse line expressing an antisense myelin basic protein (MBP) cDNA, marked decreases in both MBP mRNA and protein levels were observed, localisation of MBP in neuronal tissues from these animals, using antibody

probes revealed MBP was distributed in a distinctly non-uniform fashion (Katsuki *et al*, 1988). This is consistent with sectored *trans* inactivation of endogenous MBP expression through co-suppression. Furthermore inhibition of marker gene expression by sense constructs has been observed in transient assays in mammalian cells (Cameron and Jennings, 1991).

The aims of this programme are:

1. To establish whether co-suppression occurs in mammalian systems with an aim to obtaining dominant positions with intellectual property.

2. To define approaches to manipulate the process *in vivo* with an aim to establishing techniques to create cell lines or whole animals which are viral immune or display complete *trans* inactivation of targeted sequences.

Opportunities and Outcomes

We believe manipulation of co-suppression in animals offers novel strategies to enhance the potential applications of gene transfer into animals. Applications of such technologies might include:

- For whole animals, viral immune strains could be created or the expression of specific genes completely inactivated. The latter obviates the use of ES cells currently thought to be necessary to achieve this goal.

- Somatic cells, such as haematopoietic stem cells, could be rendered immune to viruses, a particularly potent approach for controlling viruses which infect blood cells.

- The expression of genes associated with various diseases, such as some cancers, could similarly be blocked.

Very short RNA sequences (14 bp) are capable of co-suppressing gene expression in plants (Brusslan and Tobin, 1995) and emerging data suggests that relatively short specific sequences are targeted by co-suppression. If sequences capable of eliciting co-suppression can be delivered ectopically, it might prove possible to develop novel novel nucleotide-based therapeutic agents.

- A detailed understanding of the molecular mechanism of co-suppression offers the potential to design new types of drugs.

The outcome of this programme will be generic patents covering animal and human transgenesis. Perhaps more importantly patents covering the design of novel therapeutic agents might also be developed.

Risks

We feel we possess both the intellectual and technical resources to make rapid progress with this research. Co-suppression has become a topic of major interest in plant research and whilst we are not aware of any groups extending this work to animals, the emerging interest in the area suggests such such work is likely to commence. Competition is therefore a consideration.

It is possible that co-suppression occurs only in plants. Although we do not believe this is the case, if we fail to demonstrate its existence the programme will be terminated.

Research Rationale

The complex behaviour of co-suppression in plants indicate that careful experimental design will be critical for successfully detecting the process in mammals. Two important aspects which must be considered are the frequency of co-suppression and the complex sectored nature of co-suppressed phenotypes.

In plants many independent transgenic lines are frequently constructed but only a few lines show extreme co-suppressed phenotypes. The frequency of stable phenotypes varies considerably between constructs but is often quite low, of the order of one per cent. Typically for mammalian systems only a few transgenic events are analysed, we feel that success will require screening of large numbers of transformed cell lines. Mammalian tissue culture systems offer the ability to generate large numbers of transformation events, most experiments will therefore use such systems. For whole animal experiments large numbers of animals will be created, only those showing extreme phenotypes will be maintained as lines.

Another critical consideration is the unstable sectored nature of co-suppressed phenotypes. Co-suppression would be extremely to detect if biochemical or molecular markers were analysed since variable intermediate values would be anticipated from sectored gene inactivation events. For this reason we have chosen to analyse easily scored markers. Viral immunity in tissue culture offers particular advantages since it is an easily selected phenotype. In other experiments we will use easily scored visible markers which can be readily used to score unstable sectored gene inactivation events.

Research Programme

An outline of the research project and projected timescales is shown in Figure 1. There are two broad aims:

Objective 1: To establish the existence of co-suppression in mammals.

To increase the likelihood of success and establish suitable models for the later stages of the programme three independent strategies will be pursued:

1.1 Create viral immune lines by expressing viral sequences in stably transformed cell lines.

We will use lytic viruses for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. We will conduct parallel experiments using two unrelated viruses, a simple single stranded RNA virus (Sinbis alphavirus) and a complex double stranded DNA virus, Herpes Simplex Virus I (HSV I). Both viruses are very well characterised and isolates, clones, cell lines and expertise with their manipulation are available to us.

Mammalian cell lines will be transformed with constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV) promoter. Sequences to be expressed will include specific constructs driving viral replicase genes and random "shotgun" libraries which will express all virus sequences.

For viral polymerase constructs large numbers (approximately 100) of transformed cell lines will be generated then infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines will be generated and screened in bulk for viral immunity.

Any lines obtained from such experiments will be used to more precisely define molecular and biochemical characteristics of co-suppression as outlined in Objective 2.

1.2 Inactivate the expression of nuclear genes using a simple visual reporter system.

To create a simple visual reporter we will stably transform cell lines with constructs consisting of two genes, one will express a *trans*-regulatory protein which will normally repress the expression of the second gene specifying a simple visual marker gene, the green fluorescent protein (GFP). To detect co-suppression we will target the repressor for inactivation, as a consequence GFP expression will be induced which can be easily assayed visually. To increase the likelihood of success we will prepare two sets of constructs using two different repressors, *lac* (Figge *et al*, 1988) and *tet* (Shockett *et al*, 1995). Expertise with these systems are available in house.

Cell lines will be transformed with these marker constructs. Cloned lines will be selected which show little or no background expression of GFP, but high levels of expression when induced by either IPTG (for *lac*) or the removal of tetracycline (for *tet*). Once characterised lines are established these could then be supertransformed with constructs expressing repressor sequences. Co-suppression could be simply monitored visually and co-suppressed lines purified for detailed analysis as described below.

Cell lines with such easily scored markers might also provide ideal systems for examining the effects of transient delivery of constructs as either gene cassettes, by using viral delivery systems or by direct delivery of oligonucleotide or oligoribonucleotides.

1.3 Inactivating pigment biosynthesis in transgenic mice.

To investigate co-suppression in transgenic animals we will target inactivation of pigment biosynthesis in transgenic mice. Pigment production in mice is well characterised genetically (Jackson, 1995), by targeting a single gene, tyrosinase, pigment production can be completely inhibited. This provides a simple visual assay, albinism in black mice, but more importantly sectored gene inactivation events could be easily detected. Furthermore since melanocytes can be readily cultured from mature animals this system offers the ability to undertake molecular analysis of gene inactivation events.

Constructs using the CMV promoter driving tyrosinase cDNA constructs will be prepared and used to micro-inject mouse embryos. Gene inactivation events will be monitored visually and animals showing evidence of extreme albinism will be used to establish lines. Melanocytes will be culture from such lines which will be subjected to detailed analyses as outlined below.

If no evidence for co-suppression is obtained from these experiments the programme will be terminated.

Objective 2: Molecular and biochemical characterisation of co-suppression in mammals.

Material developed from Objective 1 will potentially provide systems to study mammalian co-suppression and viral immunity using both transient and stable transformation of mammalian cell lines as well as whole animal systems. These resources will be used to undertake a detailed molecular and biochemical analyses of co-suppression. The aims of these experiments are to develop techniques which will allow the targeted inactivation of viral or nuclear RNAs at high frequency.

Cell lines obtained from 1.1, 1.2 and/ or 1.3 will be characterised to determine molecular characteristics of co-suppression. Studies will concentrate on defining molecular characteristics

of co-suppression, purifying components involved in the sequence-specific destabilisation of RNAs and developing improved strategies to control the process.

2.1 Molecular characterisation of co-suppression.

Any sequences, including sequences isolated from shotgun strategies, will be re-tested to confirm their effectiveness. Transgene expression in co-suppressed lines will be examined using Northern blots and nuclear run-ons to determine whether gene inactivation occurs posttranscriptionally as seen in plant systems. Since multiple integrations correlate with co-suppression in plant systems.Southern blots will be used to determine any influence of transgene copy number.

In plants emerging evidence indicates that quite small sequences are targeted by co-suppression. The stability of various deletion and/or chimeric RNA sequences will be analysed in co-suppressed cell lines with a specific aim of defining precisely those sequences recognised by the RNA degradative process. The compilation of such data using a number of systems might suggest design rules for targeting particular RNAs.

2.2 Biochemical analysis of co-suppression.

A central issue with co-suppression is the basis of sequence specificity. Watson-Crick base pairing must be involved, therefore some form of nucleic acid must determine this specificity. The establishment of stable co-suppressed lines will offer the opportunity to purify those components involved in specifying the destruction of particular RNAs. An *in vitro* assay for RNA stability will be developed and use as the basis for purification.

An *in vitr* assay for sequence-specific RNA degradation will be established. Using this assay factors which confer sequence specificity and are unique to co-suppressed lines will be purified. Particular emphasis will be placed on defining any nucleic acids that might co-purify with such activities since such molecules presumably determine specificity and are therefore potential targets for manipulation.

2.3 Optimising co-suppression in mammals.

To effectively manipulate co-suppression in mammalian systems approaches that result in complete, stable gene inactivation at a high frequency must be developed.

It is anticipated that experiments 2.1 and 2.2 will provide rational approaches to specifically target sequences to achieve such an objective. Moreover ongoing experiments using viral resistance in plants indicate that novel types of constructs can be prepared which yield a higher frequency of stable phenotypes (M. Graham; unpublished data). We are currently extending this work and would anticipate that improved design rules applicable to mammalian systems will emerge from this work. Factors being examined include the use of multi gene constructs, the use direct and inverted sequences and the design and use of RNA stabilising sequences.

Money

2 Research scientists, 2 research assistants x 3 years
The production of transgenic mice to be contracted out, $60,000
Patenting costs.

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Shockett, P., Difilippantonio, M., Hellman, N. and Schatz, D.G. (1995). A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* 92, 6522-6526.

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EXHIBIT 6

Title of Experiment

Interrupted palindrome

nued From
#: 50
#: 25

1. Minipep of single colonies of Cla pBC PVY.LNYV + PVY (19 tubes) prepared on 11.3.98

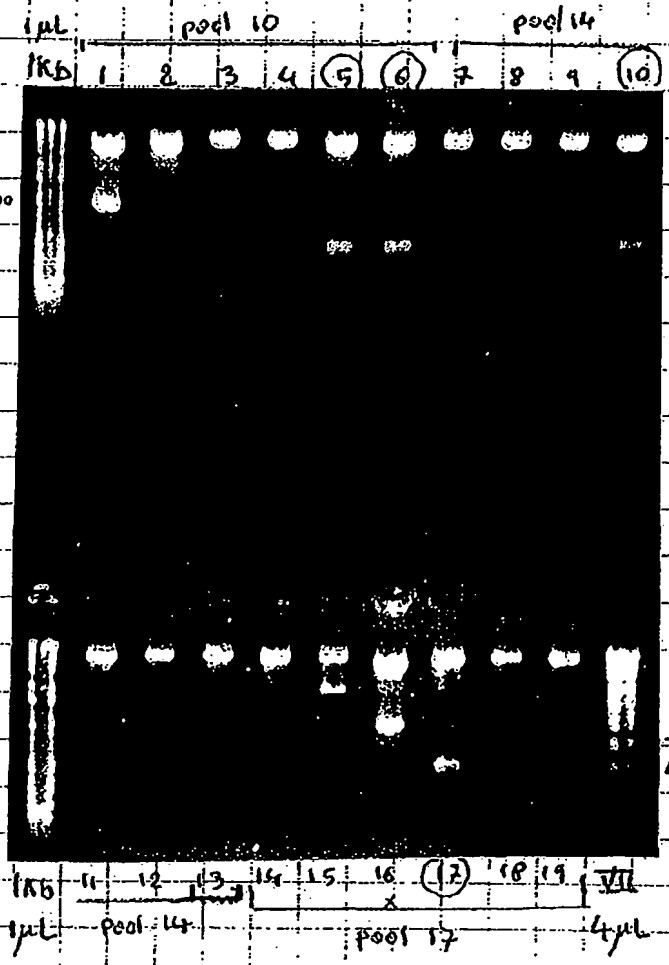
DNA extracted, and resuspended in 100µL DDW. Cut for orientation of PVY

Digestion:

DNA	5µL		Stock
buffer B	2	15µL of stock	40µL
Hind III	1		20
DDW	12		240

37°C for 1h 30min

Gel : 8µL DNA digest, 94 volts. Hind III cuts.



Singles 5, 6, 10 and 17 might have the PVY band in right orientation. Confirm with cut for both Hind III and PstI/xbaI to show presence and right orientat of the second PVY inserted

ion
53
25

Date

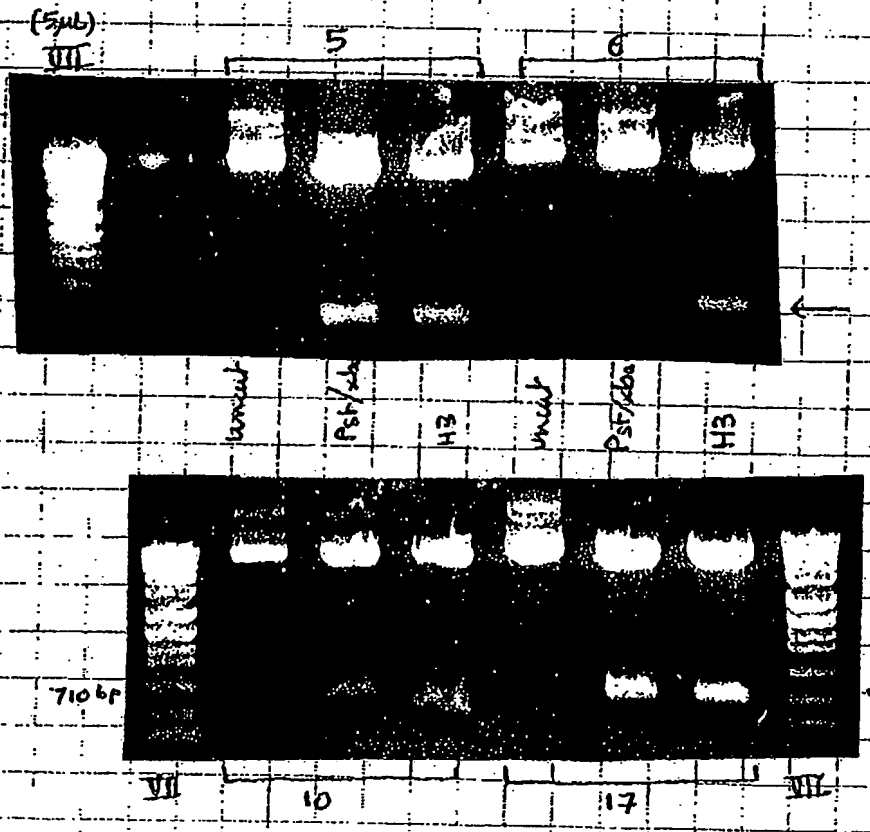
Continued From Page #: 52 Book #: 25

1. Digestion of Cla pBC PVY LNYV + PVY ; mini (Singles) No. 5, 6, 10 and 17, both for HindIII cuts, and PstI / xbaI cuts.

DNA	5 µL	5 µL
Buffer B	—	2
Buffer H	2	—
HindIII	—	1
PstI	1	—
xbaI	1	—
DDW	11	12

37°C for 3 h 20 min

Gel : loaded side by side uncut plasmid, PstI/xbaI cut and HindIII cut uncut plasmids loaded as : 4 µL DNA + 16 µL DDW + 8 µL LBuffer



PstI/xbaI fragment is smaller than the PVY band should be (750 bp) for all colonies tested

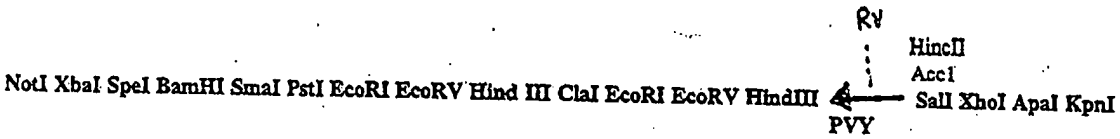
2. Sequencing of Cla pBC PVY LNYV (#9)

Continued on Page #: 54 Book #: 25

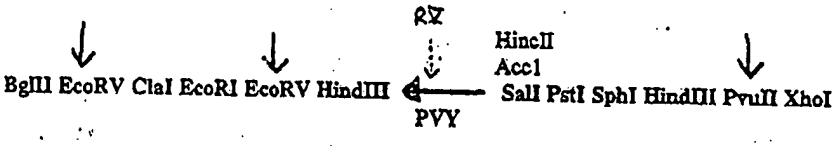
Person Conducting Experiment | First Witness of Experiment | Second Witness of Experiment

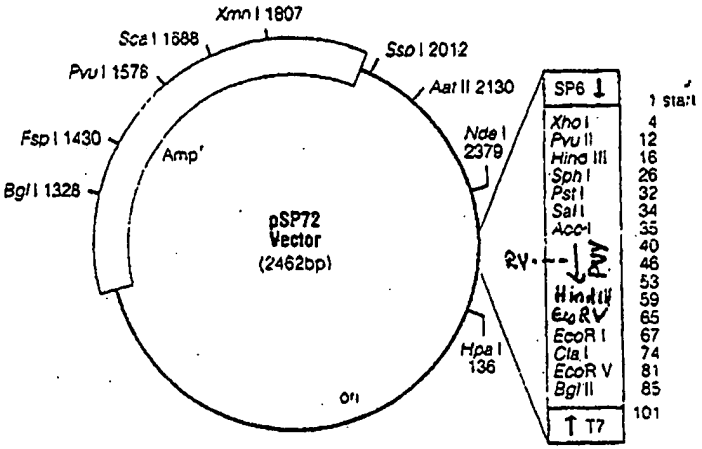
Redo digestion, still cutting Cla pBC.PVY.LNYV with SmaI, but cutting pSP72.PVY with PvuII/EcoRV, then ligate and transform. Might still have problem with HincII/SalI/AccI site

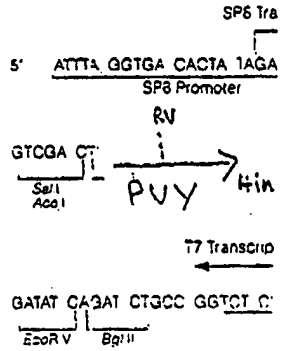
Cla pBC.PVY (ClaI/SalI pSP72.PVY into ClaI/SalI pBC.SK+)



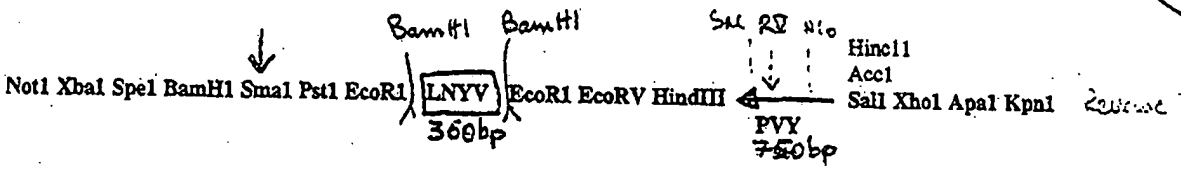
pSP72.PVY (RI/SalI pBC.PVY into RI/SalI pSP72)



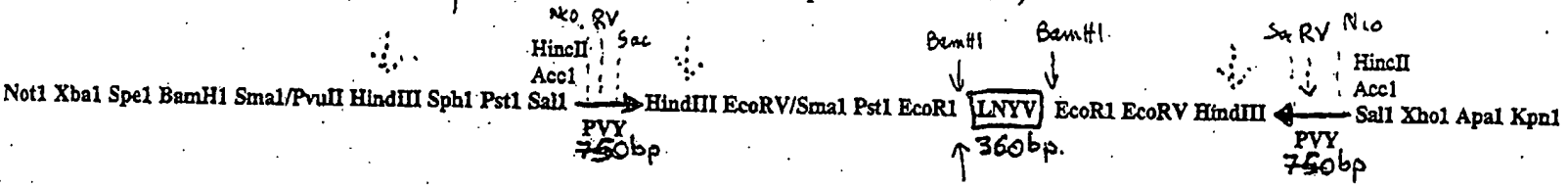




Cla.pBC.PVY.LNYV (EcoR1 pCR2.1.LNYV 4b into EcoR1 Cla pBC.PVY)

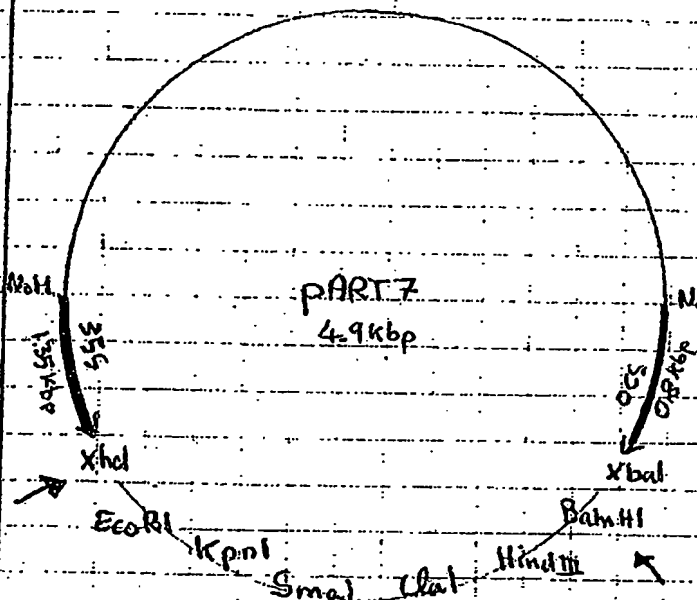


Cla.pBC.PVY.LNYV.PVY (PvuII/~~EcoRV~~ pSP72 into SmaI Cla.pBC.PVY.LNYV)



3. Prepared singles for dimers pools 2 and 4
and for timers pools 6 and 8

24 tubes LB with Ampicillin selection (in pART7)



Cla pBC.PVY (AccI/ClaI pSP72.PVY into AccI Cla pBC.PVY) (dimers)

NotI ~~EagXI~~ XbaI SpeI BamHI SmaI PstI EcoRI EcoRV ClaI EcoRI EcoRV HindIII ← Acc1/ClaI EcoRI EcoRV HindIII
(Sac NcO)
PVY
H3

Cla pBC.PVY (AccI/ClaI pSP72.PVY into AccI Cla pBC.PVY) (trimers)

NotI ~~EagXI~~ XbaI SpeI BamHI SmaI PstI EcoRI EcoRV ClaI EcoRI EcoRV HindIII ← Acc1/ClaI EcoRI EcoRV HindIII ← Acc1/ClaI
(Sac NcO)
PVY
PVY
..... XhoI ApaI KpnI
H3

PVY inserts will be in this direction ← when cut with BamHI / xhoI

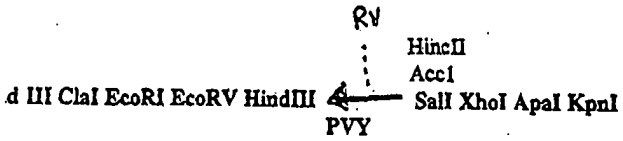
They will be in this direction → if cut with KpnI / xbaI

Continued on
Page #: 56
Book #: 25

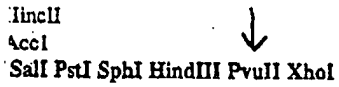
Page 54 | Date | Continued From Page #: 53 | Book #: 25 | Title of Experiment: Interrupted

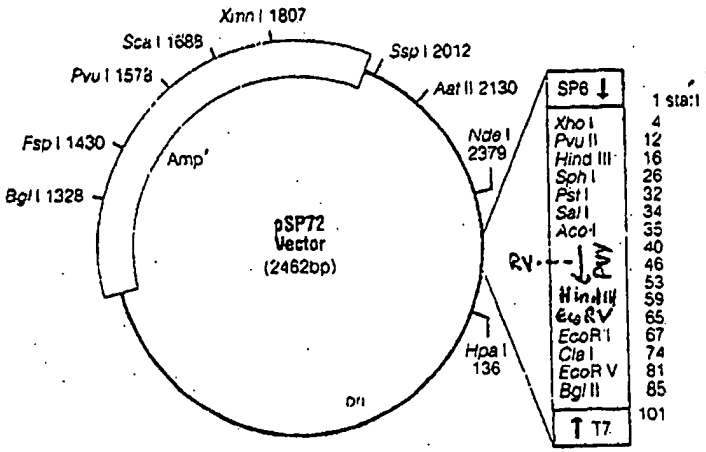
pBC.PVY.LNYV with SmaI, but cutting pSP72.PVY with PvuII/EcoRV, then shall have problem with HincII/SalI/AccI site

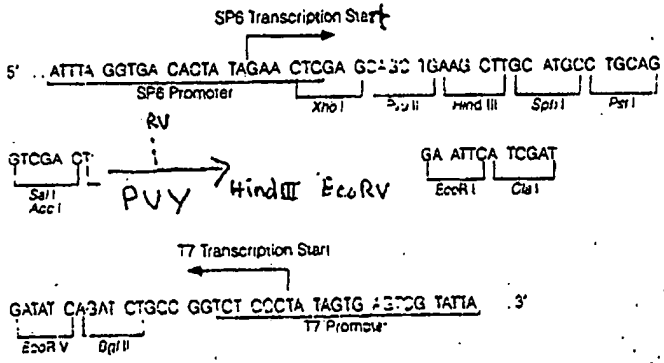
to ClaI/SalI pBC.SK+)



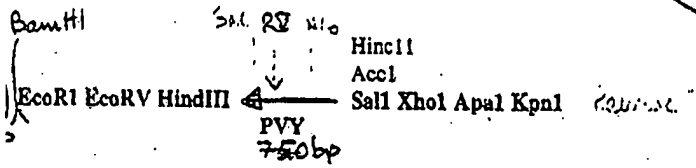
SalI pSP72)



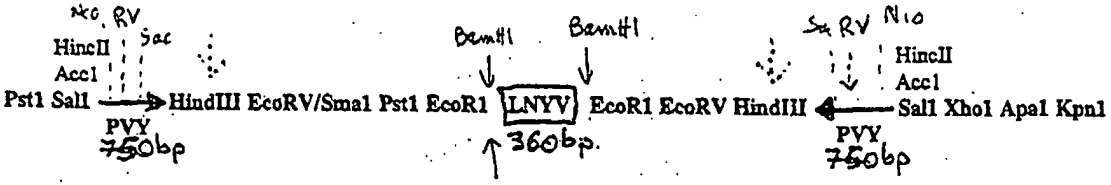




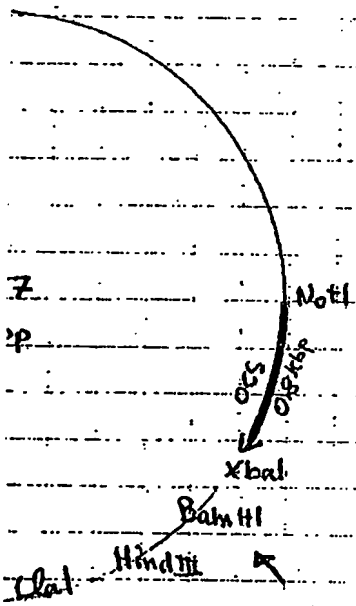
LNYV 4b into EcoR1 Cla pBC.PVY)



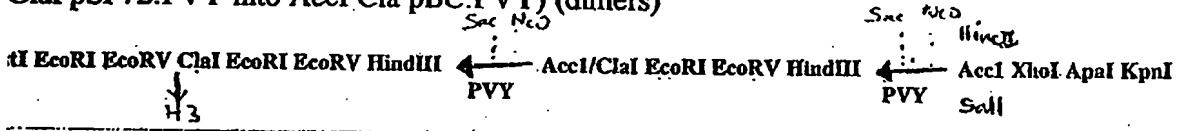
RV pSP72 into Sma1 Cla.pBC.PVY.LNYV)



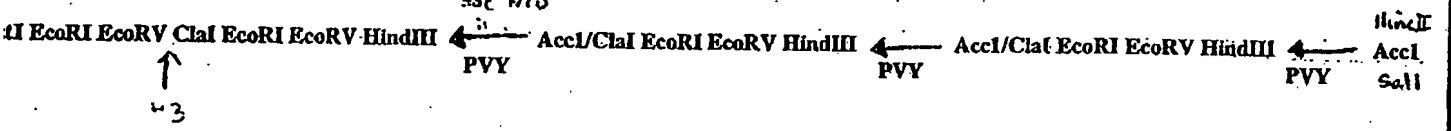
Ampicillin selection (in JRT7)



ClaI pSP72.PVY into AccI Cla pBC.PVY) (dimers)



AccI/ClaI pSP72.PVY into AccI Cla pBC.PVY) (trimers)



in this direction ← when cut with BamHI/XhoI .

direction → if cut with KpnI/XbaI .

Continued on
Page #: 56
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First Witness of Experiment

Second Witness of Experiment

Signed / / Dated

Signed / / Dated

EXHIBIT 7

Preparation of BEV constructs

Date 6/1/98

Continued From Page #: — Book #:

Overall

1) to use Bovine enterovirus as a model system to study co suppression in mammalian cells.

2) to prepare constructs containing polymerase from BEV

3) to transfect mammalian cell line with constructs probably use Madin-Darby Bovine kidney (MDBk) endothelial cell line

4) to challenge cells with BEV

Constructs to design:

1) BEV polymerase : GFP fusion → do first (Parelle has vector) use pEGFP-N1 (Clonetech Fusion vector, cat # 6085-1)
2) BEV polymerase without fusion (stop codon)

3) double promoter and terminator
- not SV40
sense
antisense

4) non-translatable (not a GFP fusion)

Other mammalian promoters: SV40
CMV
~~RSV~~
TK

Firstly need:
BEV sequence from Fiona McCarthy
↓ 4kb done in PCR2.1 vector.
designed primers with Mlds
ordered 9/12/98 from CMCB

Continued on Page #: 2 Book #: 32

Witness of Explaining Experiment	First Witness of Experiment		Second Witness of Experiment	
Heard	V Hardy		M. Graham	
Read 6/1/98 Dated	V Hardy Signed	6/1/98 Dated	Signed	2/2/98 Dated

Page 2

Title of Experiment

1. Primer design for BEV polymerase

Date 7/1/98

Continued From Page #: 1 Book #: 32

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM

Sequence Range: 1 to 1471

BEV-1 5'GGG CAG ATG TAA CAA TGG CA

F. BEV-5

Continued on Page #: 3 Book #: 32

Name of Person Conducting Experiment: M. Bernard

First Witness of Experiment: V. Hardy

Second Witness of Experiment: M. Graham

M. Bernard 7/1/98 (Signed / Dated)

V. Hardy 7/1/98 (Signed / Dated)

M. Graham 2 (Signed / Dated)

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM
Page 3

```
                1340                1360                1380
CCGTCGGGCG GGCCTTGCAT CTACCTTCAT TTAAGGCCCT CGAAAGCAAG TGGTACGATT
GGCAGCCCGC CCGGAACGTA GATGGAAGTA AATTCCGGGA GCTTTCGTTC ACCATGCTAA
ProValGlyArg AlaLeuHis LeuPheSer PheLysAlaLeu GluArgLys TrpTyrAsp>

                1400                1420                1440
CTTTGTAAAT TGCCAACTTG ATGATCCGGT TTAATTAGCT TCAATTTGGC CTGAATACAG
GAAACATTTA ACGGTTGAAC TACTAGGCCA AATTAATCGA AGTTAAACCG GACTTATGTC
SerPhe***Ile AlaAsnLeu MetIleArg PheAsn***Leu GlnPheGly LeuAsnThr>

                1460
CCACCGGATG GGGTGTAAAA AAAAAAAAAA A
GGTGGCCTAC CCCACATTTT TTTTTTTTTT T
ProThrGlyTrp GlyValLys LysLysLys XXX>
```

GA GGATCCCGGG
BamHI
CT CCTAGG G CCC 5' BEV-2

Need to design primers with restriction sites compatible with MCS in pEGFP-N1 (see p. 4 for map)
↓
looked at restriction cut site map of BEV pol
↓
decided to use BglII and BamHI sites in pEGFP-N1 which are not present in BEV pol.
↓
when designed primers, extra sites added plus kept in frame with GFP fusion

see p. 5 for primer sequences

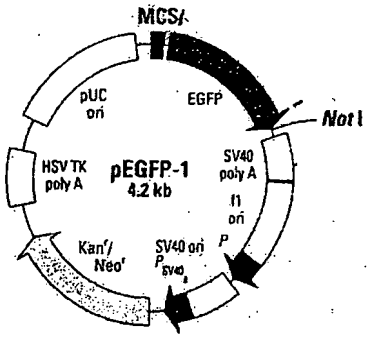
also note that selection of translation-initiation site sequence is from paper by Fütterer & Hohn (1996) Plant Mol Biol. 159-189

AACAATGGC → included in primer sequence
suggested most frequent AUG context of initiation region in both plant & mammalian genes

Continued on
Page #: 4
Book #: 32

Witnessing Experiment	First Witness of Experiment		Second Witness of Experiment	
[illegible]nard	V. Hardy		M. Graham	
[illegible]nard 7/1/98	V. Hardy	7/1/98	[signature]	7/2/98
Dated	Signed	Dated	Signed	Dated

pEGFP-1 Vector



MCS

TA GCG CTA CCG GAC TCA GAT CTC GAG CTC AAG CTT
Eco47 III *Bgl II* *Xho I* *Sac I* *Hind III* *Ecl136 II*

CGA ATT CTG CAG TCG ACG GTA CCG CGG GCC CGG GAT CC
EcoR I *Pst I* *Sal I* *Acc I* *Kpn I* *Asp718 I* *Apa I* *Sac II* *Bsp120 I* *Xma I* *Sma I* *BamH I*

EGFP
A CCG GTC GCC ACC ATG GTG
Age I

Product	Size	Cat.#
pEGFP-1 Vector	20 µg	6085-1

Expression vector that encodes the EGFP (1, 2) variant for monitoring the activity of promoters cloned into the MCS (see the GFP introduction on pages 111–112 for further information about EGFP). Sequences flanking the EGFP gene have been converted to a Kozak consensus translation initiation signal to further increase the translation efficiency in eukaryotic cells. The vector backbone provides an SV40 origin of replication and polyadenylation sequence, and a neomycin resistance cassette for selection of stably transformed mammalian cells. An f1 origin of replication allows single-stranded DNA production, and a pUC origin and kanamycin resistance gene allow propagation and selection, respectively, in *E. coli*.

pEGFP-1 is provided with a complete vector information packet (PT3026-5) and the Living Colors User Manual (PT2040-1).

UNIQUE CLONING SITES

Eco47 III, *Bgl* II, *Xbo* I, *Sac* I, *Ecl*136 II, *Hind* III, *EcoR* I, *Pst* I, *Sal* I, *Acc* I, *Asp*718 I, *Kpn* I, *Sac* II, *Apa* I, *Bsp*120 I, *Xma* I, *Sma* I, *Bam*H I, *Age* I

GENBANK ACCESSION #: U55761

REFERENCES

1. Cormack, B. P. *et al.* (1996) *Gene* 173:33–38.
2. Yang, T. T. *et al.* (1996) *Nucleic Acids Res.* 24(22):4592–4593.

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
M Bernard 7/1/98	V Hardy 7/1/98	7/1/98
Signed Dated	Signed Dated	Signed Dated

Page 6

Title of Experiment
EXPT-2
PCR to amplify BEV polymerase

Date
7/1/98

Continued From
Page #: 5
Book #: 32

PCR reactions set up on 6.1.98

DNA template = K2577 4.2 (Qiagen miniprep DNA from Flora) 4kb clone (full sequence on file)

primers: BEV-1 and BEV-2 diluted to 1 μM stocks.

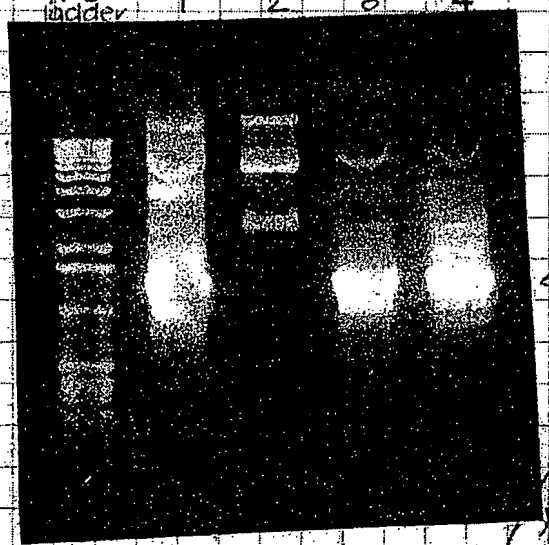
PCR rxn.	①	②	③ (1/100 of stock ① used here)	④	final conc
DNA	0.5 μl	0.5 μl	0.05 μl	0.05 μl	
1.0 μM BEV-1	2.5	2.5	2.5	2.5	0.1 μM
BEV-2	2.5	2.5	2.5	2.5	0.1 μM
10X buffer + $MgCl_2$	2.5	2.5	2.5	2.5	
10mM dNTPs	1.0	1.0	1.0	1.0	0.4mM
Taq 5U/μl	0.2	0.2	0.2	0.2*	1U
H_2O	15.8	15.8	16.25	16.25	

old tube of Taq used for ①-③ (31 Jul 97)

* new tube of Taq used (31 Jan 98)

1% TAE agarose gel: + EtBr.

500ng 1Kb ladder | PCR reactions (15μl + 2μl 10x loading buffer)

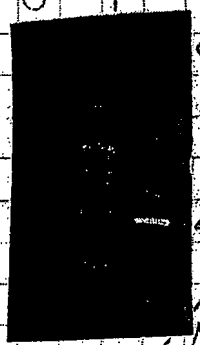


PCR conditions 9600 Perkin Elmer

Program #48 (94→47→15)
#47 94°C 0:30
60°C 0:30 } x35 cycles
72°C 1:00
#94 94°C @30
#15 15°C ∞

reactions 1, 3+4 ← 1.4kb BEV fragment for gel purification

JMB 7/1/98



aliquot of gel purified DNA on 1% MEGA

JMB 8.1.98

← 1.4kb

Gel purification: wt of each band = 0.06g
2 bands combined = 0.12g ≈ 120μl
Bresaclean DNA purification kit used (360μl Bresa-salt, 8μl Bresa-bind 3, DNA eluted in 20μl H_2O) → Ligation set up: 2μl PCR2.1 (Invitrogen) + 2μl insert (above) 1μl buffer, 1μl ligase, 4μl H_2O → 14°C O/N

Continued on
Page #: 8
Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MBernard 7/1/98	VHardy 7/1/98	2/2/98
Signed / Dated	Signed / Dated	Signed / Dated

EP 3

Preparation of DNA for cloning midipreps.

Date 7/1/98

Continued From Page #: Book #:

on 6.1.98, 1.0µl of each DNA transformed into 50µl DH5α competent cells using standard heat shock protocol, recovered in LB 1hour/37°C → transferred to 50mls LB + appropriate selection (100µg/ml)

DNA (1) pEGFP-N1 kan^R (x Darelle Thomson)

(2) K257742.2 Amp^R (in PCR2.1 vector 4kb BEV clone x Fiona)

after culture grown overnight @ 37°C, shaking

↓

30mls culture used for Bresapure midi columns

↓

DNA prepared as per kit protocol

↓

DNA resuspended in 200µl sterile MQ H_2O

↓

aliquot run on gel (~2µl)

1% TAE agarose + Ethidium bromide

JPB 1.98

Continued on Page #: Book #:

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
T. Bernard	V. Hardy	M. Cuckram
8/1/98 Dated	V Hardy Signed	8/1/98 Dated
		M Cuckram Signed
		2/2/98 Dated

Page 8

Title of Experiment: 2. cont' Transformation of PCR2.1 + 1.4kb BEV fragment

Date: 8/1/98

Continued From Page #: 6 Book #: 32

Invitrogen TA cloning kit used (TOP10F cells)
3 µl ligation from p.6
+ TOP10F competent cells
↓
30' ice, 42°C | 30", 5' ice, recover 1 hour @ 37°C, plated onto LB + ampicillin (100µg/ml)
↓
37°C O/N

8/1/98 MB

9/1/98

Colony screening of BEV clones by PCR

only 10 colonies (white), >20 blue colonies.
method:
pick colony into 10µl LB broth, vortex, quick spin, add 1µl to PCR reaction (NB. used toothpick to pick colonies and also streak colony onto plate for use with PCR master mix minis.)

		x 13
10x PCR buffer (+Mg++)	2.5 µl	32.5 µl
MQ H_2O	19.8	257.4
10mM dNTP's	0.5	6.5
10µM primer 1 (BEV-1)	0.5	6.5
2 (BEV-2)	0.5	6.5
broth	1.0	—
5U/µl Taq (Boehringer)	0.2	2.6
	25.0	312 µl (24 µl / tube)

PCR reactions 1–10 (clones 1–10)
11 broth control
12 blue colony (negative control)
13 original DNA template PCR2.1 + BEV 4.1kb

Program 48 (changed from p.6) now #94 → #47 → #72 → #15

94°C	90"	
94°C	30"	} x35 cycles
55°C	30"	
72°C	1'	
72°C	5'	15°C ∞

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MBernard 9/1/98	VHardy 9/1/98	2/9/98
Signed / Dated	Signed / Dated	Signed / Dated

BEV-1 and BEV-2 primer sequences

Date 7/1/98

Continued From Page #: 4 Book #: 32

- 394 Synthesis Setup Listing - (Version 2.01)

Column 1 2:29:50P , 9/12/97

Run ID : BEV-1 ca 250µg
Cycle : DS 40NM
End Proc: DSCESS (DMT = Off)
Sequence: 448497B

Total bases = 38
A= 14, G= 9, C= 9, T= 6, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 11705.6

START
5'> CGG CAG ATC TAA CAA TGG CAG GAC AAA TCG AGT ACA TC <3'
Bgl II initiation region

Column 2 2:29:51P , 9/12/97

Run ID : BEV-2 92 mg purified at no extra cost.
Cycle : DS 40NM
End Proc: DSCESS (DMT = ~~Off~~ on)
Sequence: 448597B

Total bases = 31
A= 8, G= 6, C= 11, T= 6, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 9420.2

5'> CCC GGG ATC CTC GAA AGA ATC GTA CCA CTT C <3'
BamHI

Calculation of primer concentrations: (primers resuspended in 100µl sterile H_2O)

BEV-1 $\frac{250\mu g / 100\mu l}{11705.6} = 2.14 \times 10^{-4}$ M

$= 214\mu M$

BEV-2 $\frac{92\mu g / 100\mu l}{9420.2} = 9.77 \times 10^{-5}$ M

$= 97.7\mu M$

10µM working stocks prepared:

10µl BEV-1 @ 214µM + 204µl MQ H_2O

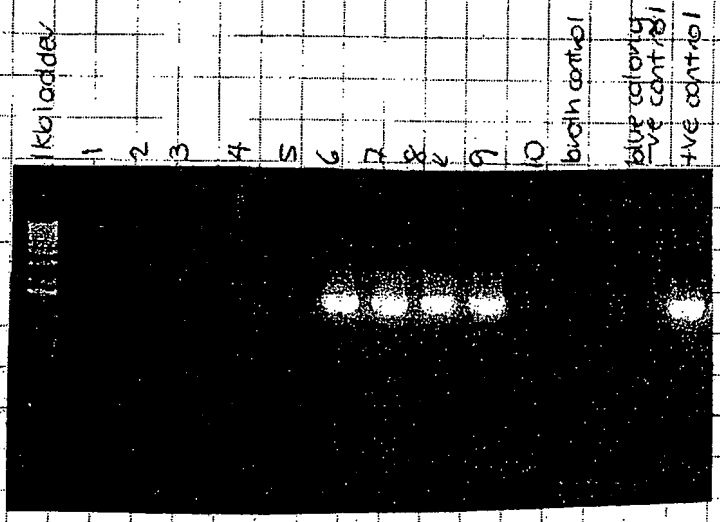
10µl BEV-2 @ 98µM + 88µl MQ H_2O

Continued on Page #: 6 Book #: 32

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
[illegible]	V. Hardy	M. Graham
Dated 7/1/98	Signed V. Hardy	Dated 7/1/98 Signed M. Graham Dated 7/2/98

Project: PCR2.1 + 1.4kb BEV polymerase results.

PCR/colony screening results of PCR2.1+1.4kb BEV



← 1.4kb BEV polymerase band

clone # 1 2 3 4
+ + + +

4 positive clones with PCR and BEV-1 and BEV-2 primers: set up miniprep cultures of clones 6, 7, 8, 9

MPB 9/1/98

8/1/98

8ml LB broth + kanamycin (100µg/ml) + colony streak of 4 positive clones
↓
37°C O/N MPB 11/1/98

9/1/98

cultures have grown ok., no time to prepare minis today ... 1.5ml of each culture spun down to collect & freeze pellet. Glycerol stocks prepared

MPB 12/1/98

Continued on Page #: Book #:

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
S. Bernard	V. Hardy	M. Graham
MPB Signed	12/1/98 Dated	V Hardy Signed	12/1/98 Dated	M Graham Signed	2/2/98 Dated

Page 10

Title of Experiment: 2.0ml Minipreps on PCR positive clones of PCR2.1+1.4Kb BEV

Date: 13/1/98

Continued From Page #: 9 Book #: 32

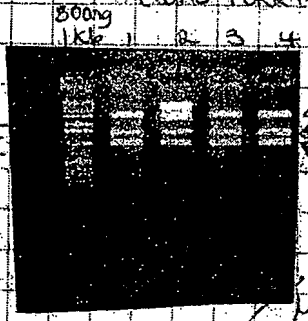
Qiagen mini spin kit used with bacterial pellets from 2ml eluted in 100µl elution buffer supplied.

BglII / BamHI digests on minis

		×4
DNA	5.0µl	-
10x buffer B	2.0	8.0
BglII	0.5	2.0
BamHI	0.5	2.0
H_2O	12.0	48.0
total	20.0	60.0

→15µl/tube, added DNA, incubated 37°C 1.5hrs

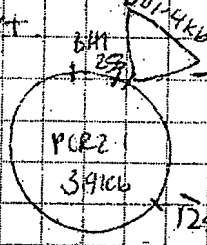
Total digest run on 1.5% TAE gel 90V/35' stained in ethidium bromide



← 3.0Kb vector band
← 1.4Kb band = BEV BglII/BamHI fragment
vector band 1Kb

1kb 6/1/98

Comments:
vector PCR2.1 has a BglII and BamHI site, positions 1243 and 253 respectively.. expect to see another 2 bands along with the insert.
PCR2.1 = 3.9Kb
insert band (BEV) 1.4
vector bands 3.0 } ~4.0Kb
1.0 }



Next:
select clone for further work (clone #1)
set up digests to make pEGFP-N1 + BEV

Continued on Page #: 12 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Cochran
Signed: mb	Signed: VHardy	Signed: MC
Dated: 13/1/98	Dated: 13/1/98	Dated: 20/1/98

Cloning of 1.4kb BEV fragment into pEGFP-N1

Date 14/1/98

Continued From Page #: Book #:

Aim: to digest pEGFP-N1 midiprep DNA with BamHI and BglII and use BamHI/BglII BEV polymerase as an insert.

Method:

Digests

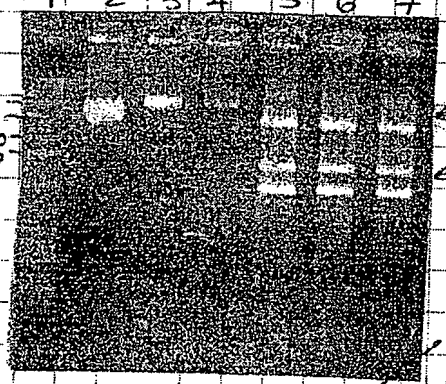
PCR2.1-BEV clone#1	15.0 µl	pEGFP-N1 midi	6.0 µl
10x buffer M	5.0	10x buffer M	2.5
Boehringer H_2O	28.0	BglII	1.0
BglII	1.0	BamHI	1.0
BamHI	1.0	H_2O	14.5
	50.0		25.0

- incubated 37°C / 1.5hrs, 1.5% TAE agarose gel
- total digest of BEV clone#1 run on gel over 3 lanes
- aliquot of pEGFP run on gel.

lane 1 500ng 1Kb
2 2µl pEGFP-N1 uncut midi
3 2µl pEGFP-N1 digest
4 2µl PCR2.1-BEV#1 uncut midi
5 18µl PCR2.1-BEV#1 digest
6 "
7 "

→ need to treat digest with phosphatase as BglII and BamHI are compatible ∴ will religate.

BglII: A/GATCT / TCTAG/A
BamHI: G/GATCC / CCTAG/G
G/GATCT BglII
BamHI CCTAG/A



← 4.2Kb vector band

← 1.4Kb BEV bands cut out for gel purification and stored @ 4°C.

MB 14/1/98

Continued on Page #: 12 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
#1. Bernard	V. Hardy	M. Carlson
Dated 14/1/98	Signed 14/1/98 Dated	2pgs

Title of Experiment
Expt. 4 cont.
Cloning of 1.4kb BEV into pEGFP-N1 cont

Date
15/1/98

Continued From
Page #: 11
Book #: 32

On 15/1/98
Preparation of more insert, as tube from yesterday misplaced.

PCR2.1+BEV #1	20.0µl
10x buffer M	6.0
Bgl II	1.0
BamHI	1.0
H_2O	23.0
	50.0

incubated 37°C/1.5hours
digest run on 1.5% TAE gel 80V/35'
also found old tube!

Gel purification : see below

JMB
15/1/98

Phosphatase treatment of pEGFP-N1 BglII/BamHI:
estimation of conc. = 0.25µg/µl
= 250ng/µl
use 1/2 of digest ~10µl = 2.5µg
2.5µg/3.04 = 5.1pmol of ends
4.2kb 0.01units SAP/pmol ends
Shrimp Alkaline Phosphatase (SAP) (Amersham) 1U/µl → 1:10 dilution to 0.1U/µl
10µl DNA + 5µl 10x Reaction Buffer + 1µl 1:10 SAP + 34µl H_2O = total 50µl
37°C/1hour, 65°C/20' → aliquot run on gel purification gel/p.13

Gel purification using Bresapure DNA purification kit:

		Amount of Bresa-salt
wt. of 1.4kb BEV insert bands + gel from yesterday	= 0.25g = 250µl	750µl
from today	= 0.22g = 220µl	660µl

6µl Bresa-bind used for each tube
DNA eluted in 15µl MQ H_2O
Aliquot run on 1.5% TAE

Gel result on p.13

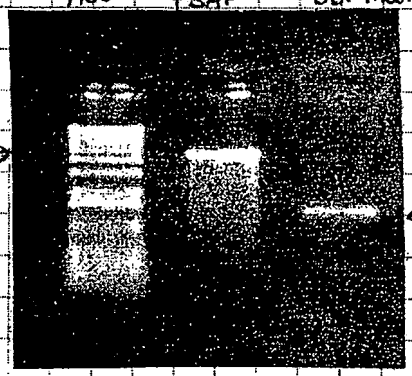
Continued on
Page #: 13
Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
Signed: MMB	Dated: 15/1/98	Signed: V Hardy
Dated: 16/1/98	Signed:	Dated: 21/1/98

Cloning of 1.4kb BEV into pEGFP-N1 cont.

Purification of pEGFP-N1 BamHII/BglII fragment SAP treated: (using Bresapure DNA purification kit)
wt. of ~~band + gel~~ DNA + solution = 50µl 7µl Bresa-bind, 150µl bresa-salt
eluted in 15µl H_2O → aliquot run on gel.

1% TAE gel of vector and insert aliquots



1% TAE
80V / 40'
Stained in EtBr 10'

comments: smear of vector ∴ could be degradation of vector DNA?

Ligations to set up:

	VECTOR	INSERT
(1)	pEGFP-N1 BglII/BamHI −SAP 4.2kb not gel purified	1.4kb BEV gel purified
(2)	pEGFP-N1 BglII/BamHI +SAP	"
(3)	"	no insert

Ligations set up on 15/1/98 → O/N @ RT

	①	②	③
vector	1.5µl	4.5µl	4.0µl
insert	8.0	8.0	−
10x ligation buffer	1.5	1.5	1.5
Ligase	1.0	1.0	1.0
H_2O	−	−	8.5

Continued on Page #: 14 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	V. Harding	M. Graham
16/1/98 Dated	16/1/98 Signed	16/1/98 Dated
		22/1/98 Signed Dated

Page 14

Title of Experiment: Expt 4 cont.

Transformations of ligations from p.13

Date: 16/1/98

Continued From Page #: 13 Book #: 32

pEGFP-N1/BglII+BamHI + BEV polymerase/BglII/BamHI

9µl of each ligation + 50µl DH5α competent cells on ice

↓ 20' ice, heat shock 42°C/60", ice 5'

↓ added 450µl LB broth

↓ 37°C 1 hour

↓ plated 100µl + 200µl onto LB + kanamycin 100 µg/ml

↓ 37°C O/N, 4°C stored.

MJB 17/1/98

19/1/98 Transformation results: CFU (colony forming units)

1) GFP + 1.4kb BEV ⊖SAP
plate (1) >500 CFU
(2) >500 CFU

2) GFP + 1.4kb BEV ⊕SAP
plate (1) ~34 CFU
(2) ~30 CFU

3) GFP ⊕SAP no insert control
plate (1) ~40 CFU

DH5α control on LB+kan <10 CFU

Comments:
- ⊕ SAP ligation, CFU much less ∴ SAP treatment reduces efficiency of ~~ligation~~ transformation as expected
- control with no insert, just vector SAP treated shows background ∴ SAP not 100% effective.

Next: screen colonies by PCR

Continued on Page #: 15 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MJB Signed	19/1/98 Dated	VHardy Signed
19/1/98 Dated		Signed

Exp+ 4 cont.

PCR screening of putative pEGFP-N1 + BEV

Date 19/1/98

Continued From Page #: 14 Book #: 32

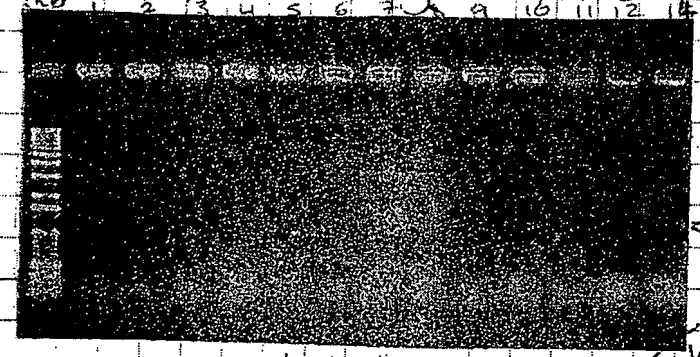
Aim: to amplify ~1.4kb band using BEV-1 and BEV-2 primers
colonies picked → transferred to 10µl LB broth

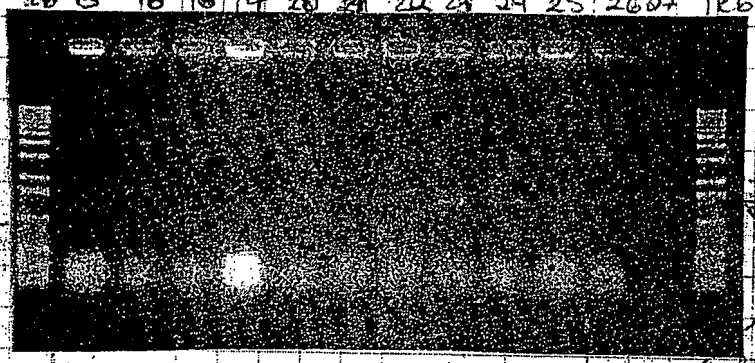
master mix		x 27
MQ H_2O	19.8µl	534.6µl
10mM dNTP's	0.5	13.5
10µM BEV-1	0.5	13.5
BEV-2	0.5	13.5
10x PCR buffer	2.5	67.5
Taq	0.2	5.4
broth	1.0	—
		648µl 24µl per tube

PCR reactions 1-12 = ligation 1 no rxn 13 or rxn 17
13-24 = ligation 2
25 = ligation 3
26 = pUR2.1:BEV colony #1
27 = -ve broth control

Program #92 (94→91→72→15) on 9600 (Animal lab)
annealing temp 60°C 35 cycles, extension 1' @ 72°C
68°C / 10' Finishing off 15°C ∞

no #12 when toothpick PCR put into LB tube. Misnumbering somewhere??





← 1.4kb faint bands (duplicates)

Comments: ??
- positive control has not worked (no plasmid control)
- faint bands with 5, 6, 7 at 1.4kb
- stronger band with 23
- minis set up from 1.4kb band 4, 5, 6, 7, 8, 22, 23 and 24

Continued on Page #: 16 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	V. Hardy	M. Graham
Bernard 19/1/98 Dated	V. Hardy 19/1/98 Signed / Dated	21/1/98 Dated

Title of Experiment EXPT 4 cont'd

Minipreps and digests on putative PEGPP + BEV clones

Date 20/1/98

Continued From Page #: 15 Book #: 32

Clones 4, 5, 6, 7, 8, 22, 23, 24
Qiagen mini spin kit
1.5ml o/n culture
eluted in 100µl elution buffer supplied

↓

next: digest with BglII and BamHI, which should release the 1.4kb BEV fragment.

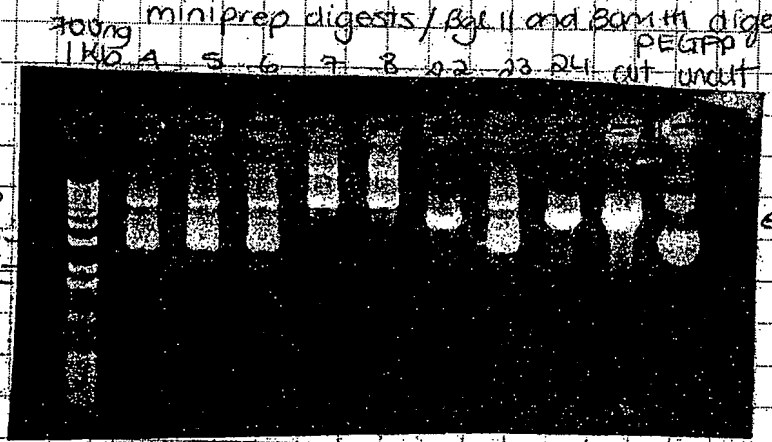
MJB 20.1.98

21/1/98 Digests on above minis

		x9
DNA	5.0µl	+
10x buffer M	2.0	18.0
BglII	0.5	4.5
BamHI	0.5	4.5
H_2O	12.0	108.0
	20.0	135.0

control PEGPP-N1 2µl DNA cut with BglII/BamHI

15.0µl/tube
+ 5.0µl DNA
37°C 1.5hrs, + 3µl loading buffer
½ of digest run on 1% TAE gel 85V/50mins.



← 4.2kb vector band

Results not as expected, no 1.4kb visible with any of the digests; ~2.8kb band (not clear) with minis 4, 5, 6 and 23, which could be a dimer of 2x1.4kb? Second band not correct size for vector. ∴ minis don't look good → set up ligation again?

Continued on Page #: 17 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MBernard 21/1/98	VHardy 21/1/98	
Signed Dated	Signed Dated	Signed Dated

EXPT. 5.

New ligation of pEGFP-N1 + BEV

Page 17

Date 21/1/98

Continued From Page #: 16 Book #: 32

Aim: to set up new ligation using less vector and more insert and some new ligase

Method:

located new source of ligase (Boehringer exp 31 Jan98) instead of previously used stock (exp. Jun 97)

Ligations

pEGFP-N1 /BglII+BamHI+CIAP (p.13)	2 µl	
1.4kb BEV/BglII+BamHI gel purified (p.13)	10.0	
10x ligation buffer	1.5	
ligase	1.0	1.5
	15.0	

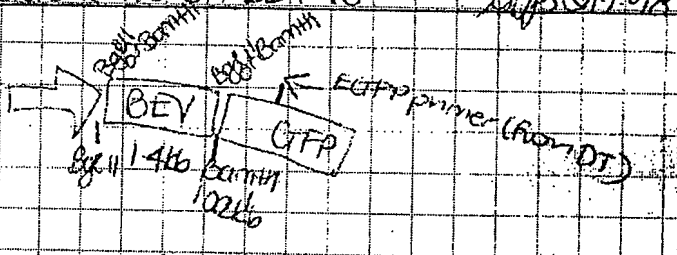
controls to check efficiency of new ligase.

	⊕ ligase	⊖ ligase
pEGFP-N1 /BglII+BamHI (p.11, lane 3)	2.0	2.0
10x ligation buffer	1.5	1.5
ligase	1.0	—
H_2O	10.5	11.5
	15.0	15.0

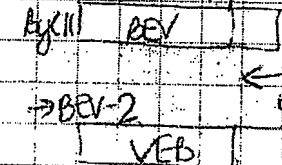
all ligations incubated O/N @ 4°C → recovered in SOC. MB 21.1.98
transformed into XL-1 blue cells on 22.1.98

PCR to check ligations

use EGFP primer (from DT) 200bp downstream from start of GFP (reverse primer) + BEV-1 or BEV-2



can expect 2 orientations →BEV-1 BamHI



forward 1.6kb fragment
reverse (antisense) 1.6kb.

Emdeer Mix	
H_2O	59.4
Buffer	7.5
dNTPs	0.75
Taq	0.6
	69 → dispensed into 11.5µl aliquots
	+ 0.25µl 10µM primer
	+ 0.5µl ligation reaction

Continued on Page #: 18 Book #: 32

Witnessing Experiment	First Witness of Experiment	Second Witness of Experiment
Read	V. Hardy	M. Graham
22/1/98 Dated	V Hardy Signed	22-1-98 Dated
		22/1/98 Dated

Page 18

Title of Experiment Expt. 8 cont.

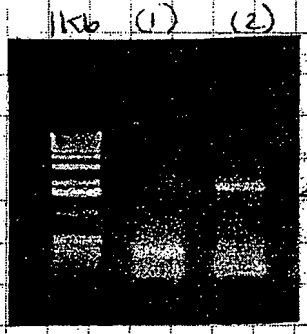
PCR results from ligation + Transformation results.

Date 23/1/98

Continued From Page #: 17 Book #: 32

PCR reaction
(1) ligation pEGFP-BEV (p.17)
(2) "

Primers
EGFP + BEV-1
EGFP + BEV-2



Result: no 1.6 kb fragment with BEV-1 primer ∴ no forward orientation recombinants
~1.6kb with BEV-2 primer reverse orientation or antisense recombinants in ligation.

Transformation results after O/N @ 37°C.

		# colonies
BEV + pEGFP	plate (1)	2 CFU / 100µl
	(2)	2 CFU / 80µl
⊕ ligase control		65 x 8 CFU / 100µl
⊖ ligase control		9 CFU / 100µl
-ve controls (x-1 blue) on	Kan	1 CFU / 100µl
	Amp	50 / 100µl

No ligation mix left as added SOC to ligation instead of x-gal ccls!!

Comments:
only 4 colonies to screen from transformation
∴ unlikely to find the clone
→ something wrong with transformation? other work done in parallel w larger no.'s colonies obtained?

Could plate out more transformation but may be better off starting again.

Decided to screen 4 colonies by PCR (BEV-1 and BEV-2 primers) + old ligations from 15-1-98

see p. 19

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
N. Bernard	V. Hardy	M. Graham
MBernard (Signed) 24/1/98 (Dated)	VHardy (Signed) 23/1/98 (Dated)	(Signed) (Dated)

Expt. Scott

PCR to check colonies + old ligation (15.1.98)

Page 19

Date 25/1/98

Continued From Page #: 18 Book #: 32

BEV master mix for colony screening

		x8
H_2O	9.9µl	79.2µl
2mM dNTPs	0.25	2.0
10µM BEV-1	0.25	2.0
BEV-2	0.25	2.0
5u Taq pol	0.1	0.8
KCl buffer	0.25	10.0
broth	0.5	—
	12.5	96 → 12µl dispensed per tube + 0.5µl broth + colony

Program 48 #94 94°C 0:30
#47 {94°C 0:30, 55°C 0:30, 72°C 1:00} x30 cycles
#72 68°C 5:00 15°C ∞

Reactions

1-4 = colonies 1-4
5 K257742 0.6µl of 1:10 dilⁿ
6 PCR2.1 + BEV clone #1 0.6µl
V vector only colony from control transformation (religated vector)
B broth only (-ve control)

ligation

pEGFP +SAP + BEV (p.13) = ligation 2

reaction		primers
1	lign 2	BEV-1 + EGFP
2	"	BEV-2 + EGFP
3	control pEGFP-N1 vector	EGFP

can't remember which other primer I used!!! MW 28/1/98

1% TAE agarose gel

ligation results: no 1.6kb fragments ∴ indicates no recombinants in this ligation. 1.6kb bands with pEGFP (larger than) 25.1.98 → may have to check again.

Results: no positive clones from colonies 1-4 (didn't really expect to find one!), controls all OK. Maybe colony PCR not working well (toothpicks causing inhibition??), as positive controls are plasmid DNA. NEXT: START AGAIN.

Continued on Page #: 20 Book #: 32

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Witnessed MW	V. Hardy	M. Graham
Dated 25/1/98	Signed V Hardy Dated 25/1/98	Signed MG Dated 2/2/98

Page 20

Title of Experiment: Expt. 5 cont

Cloning started again.... pEGFP-N1 + 1.4Kb BEV

Date: 26/1/98

Continued From Page #: 19 Book #: 32

Vector pEGFP-N1

midiprep DNA	10 µl
BM 10x buffer M	2.5
H_2O	10.5
Bgl II	1.0
BamHI	1.0
	26.0

Insert BEV from PCR2.1-BEV clone#3

DNA (miniprep #3)	10.0 µl
10X buffer M	2.5
Bgl II	1.0
BamHI	1.0
H_2O	10.5
	26.0

Incubated 37°C / 1.5hrs

Dephosphorylating vector (using shrimp alkaline phosphatase) SAP

to 25µl digest
1.0µl SAP (1U/µl) Amersham
3.0µl 10x SAP buffer
1.0µl H_2O

inc. 37°C / 1hour
heat inactivated 65°C / 15mins → ice

Total amount of vector + insert run on 1% TAE agarose gel (photo in book 8, p.59)
vector band at 4.2Kb (still alot of DNA in well?)
insert band at 1.4Kb
bands excised for gel purification

Gel purification (using QIAprep DNA purification kit) brand new kit Paul Campbell

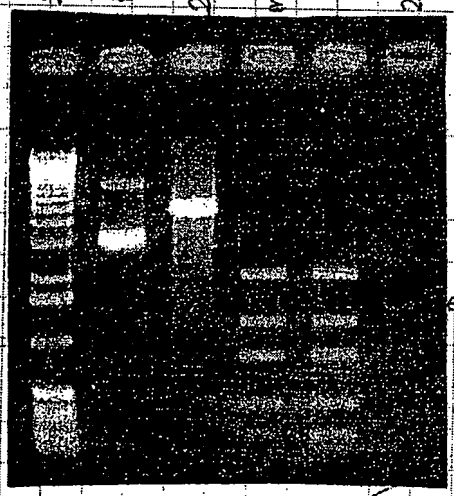
		µl QG buffer
vector	0.11g ≅ 110µl	330
insert	0.08g ≅ 80µl	240µl

both eluted from column in 30µl H_2O, speedivac low/40
aliquots run on gel (see p. 21)

Continued on Page #: 21 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Gardam
M Bernard 26/1/98	V Hardy 26/1/98	[illegible] 27/1/98
Signed / Dated	Signed / Dated	Signed / Dated

Concentrations of DNA for ligations.



Gibco-BRL low range mass ladder (amount of DNA 1µl band represents)

2µl	1µl band represents
100 ng	50ng
60	30
40	20
20	10
10	5
5	2.5

Approx. concentrations of DNA: MP 26/1/98

		vol.	yield ng	size ratio	Amt DNA 1:3 ratio
vector	≥100ng/2µl ~250ng	19µl	2375	~3	100ng
insert	10ng/2µl	20µl	100	1	100ng.

ligation ⊕ insert
- 0.5µl vector
- 16.5µl insert
- 2.0µl 10x BM buffer
- 1.0µl ligase (31 Jan 98)
- 20.0µl

inc. O/N @ RT

⊖ insert
- 0.5µl vector
- 1.0µl ligase
- 1.5µl 10x buffer
- 12.0µl H_2O

Continued on Page #: 22 Book #: 32

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
nard	V. Hardy	M. Graham
Dated 26/1/98	Signed VHardy	Dated 26/1/98
		Signed
		Dated 2-2-98

Page 22

Title of Experiment Expt. 5 cont

PCR to check ligations

Date 27/1/98

Continued From Page #: 21 Book #: 32

Aim: to check ligations and to transform ligations into XL1-blue

METHOD: PCR master mix x6

H_2O	9.94 µl	59.65
BM 10x buffer	1.25	7.5
10mM dNTPs	0.21	1.25
Taq	0.10	0.60
		69 → 11.5µl aliquots, + 0.25µl each primer (10µM stock) + 0.5µl ligation.

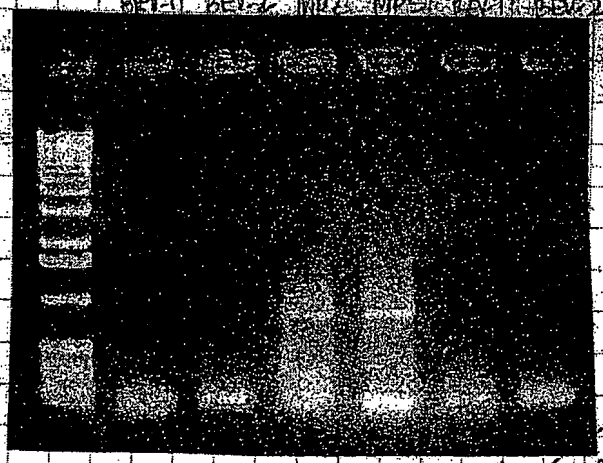
0.17mM final conc

LIGATION	10µM PRIMERS	
pEGFP + BEV	BEV 1	EGFP
	BEV 2	EGFP
pEGFP -	BEV 1	EGFP
	BEV 2	EGFP
OTHERWORK		
PSP72 del + CMV MP	MP2	T7
	MP6	T7

Program #48 20cycles/55°C

MB 27/1/98

1% TAE agarose gel, 90V/40'



no 1.6kb bands with any primer set for pEGFP +BEV ligation

Continued on Page #: Book #:

Name of Person Conducting Experiment: M Bernard — Signed: MB — Dated: 28/1/98

First Witness of Experiment: V. Hardy — Signed: V Hardy — Dated: 28/1/98

Second Witness of Experiment: M Cuckow — Signed: — Dated: 28/1/98

Expt. Transformations into XL-1 blue cells.

Date 28/1/98

Continued From Page #: 22 Book #: 32

Cm 27.1.98

competent cells - XL1-blue (chemical)

500µl cells ⇒ dispensed in 100µl aliquots

7µl of each ligation, heat shock method, recovered in 500µl SOC media 1hour/37°C.

↓

inc. 37°C O/N

	selection	# colonies
pECFP + BEV	kan	≤0
pECFP (no insert)	"	≤0
controls		
pSP72.del/ECORV + ligase	Amp	≤0
" - ligase	"	≤0
XL1-blue control	Amp	≤0
"	kan	≤0

Comments:

transformation has not worked for some reason. competent cells could be a problem, although a transformation for Vanessa performed at the same time, resulting in some colonies.

What next?!? Sequence PCR2.1+BEV clones.

Set up new ligation using already prepared vector and insert.

6.0µl	BEV insert	(1.0µl from old preparation on 15.1.98 and 5µl of newly prepared on 26.1.98)
0.5µl	vector (as on p.21)	
1.0µl	10x buffer	
1.0µl	ligase (31 Jan 98)	
1.5µl	H_2O	
10.0µl		inc. on bench for 3 hours.

Ligation desalted using Amicon microcon columns (concentration 30 No. 42418) (also desalted 26.1.98 ligation) Ligation of 10µl + 480µl H_2O, 2.5K/30mins in column, final volume ~20µl P.10.

Continued on Page #: 24 Book #: 32

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
[illegible]nard	V. Hardy	
28/1/98 Dated	V Hardy Signed	28/1/98 Dated
		M. Graham 10.2 PG Signed
		Dated

Page 24

Title of Experiment: Expt. 5 cont. Transformation of pEGFP + BEV ligation

Date: 28/1/98

Continued From Page #: 23 Book #: 32

Aim :- to transform ligation from p.23 into electrocompetent cells
- also to check transformation protocol by using pUC18 as a control

Cells:

JS4 Biorad RecA+ (from Roger Mitchell) electrocompetent
DH5α chemically competent

JS4	12.5 µl	1 µl	pUC18 (10 ng)
JS4	12.5 µl	2 µl	pEGFP + BEV (26.1.98 lign)
JS4	12.5 µl	2 µl	pEGFP + BEV (28.1.98 lign)
DH5α	20 µl	1 µl	pUC18 (10 ng)

Electroporation method:
conditions: 1.5V, 25µF, 200Ω, 0.1cm cuvette
recovery in SOC, 37°C / 50'

Heat shock:
42°C / 60", recovery in SOC / 37°C / 50'

Time constants after electroporation, all ~4.5

30-100µl plated out onto selection, inc. O/N @

#

Continued on Page #: 25 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Barnard	V. Harding	M. Graham
M Barnard 28/1/98	V Harding 28/1/98	
Signed Dated	Signed Dated	Signed Dated

Experiment Expt. Scott

Page 25

Date 2/2/98

Continued From Page #: 24 Book #: 32

Results of transformations

from 28.1.98:

TNTC = too numerous to count

plate	#colonies
2.5μl JS4 + pUC18(long)	TNTC / 100μl small + large colonies
20μl DH5α + pUC18(long)	TNTC / 80μl less than with JS4
JS4 + pEGFP+BEV (26.1.98)	≤0
JS4 + pEGFP+BEV (28.1.98)	1

Paul plated out remainder of transformation (new 4x100 plates for pUC18) & 100 plates already prepared for pEGFP ligation.

plate	#colonies
JS4 + pEGFP+BEV (26.1.98)	≤0
JS4 + pEGFP+BEV (28.1.98)	≤0

Comments: results are not very promising. pUC18 controls gave alot of colonies (unable to work out efficiency), but only one colony with pEGFP+BEV ligation from 28.1.98. Need to make more comp. cells.

Discussions with Mick → start again, don't use any SAP or gel purification. Try counterselection method.

pCR2.1+BEV clone #1 kan^R Amp^R

pEGFP kan^R

∴ choose colonies that only have kan^R.

Continued on Page #: 26 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	V. Hardy	M. Graham
Bernard 2/2/98 Dated	V Hardy Signed	2/2/98 Dated
		10/2/98 Dated

Page 26

Title of Experiment: EXPT. 6

New digests of vector + insert.

Date 2/2/98

Continued From Page #: 25 Book #: 32

Aim: to set up new ligation using no gel purification.

PCR2-1 +BEV #1 mini	19 µl	pEGFP-N1 (xDT 0.3 µg/µl)	10 µl
BM 10x buffer M	5	BM 10x buffer M	5
BM BglII	1	BM BglII	1
BM BamHI	1	BM BamHI	1
H_2O	28	H_2O	33
	50		50

inc. 37°C 12 hours

DNA ppt'd - 125 µl 100% etOH + 5 µl 3M Na acetate, pH 5.2, freeze 10', 14K / 20' @ 4°C, pellet washed in 70% etOH, pellet dried + resuspended in 10 µl H_2O.

0.8% TAE agarose gel, 100V

lane
1. 2 µl 1kb ladder
2. 1 µl digest pEGFP-N1 BglII/BamHI (14.1.98)
3. 1 µl DNA mass ladder
4. 1 µl digest pEGFP-N1 BglII/BamHI (today's)
5. 1 µl digest PCR2-1+BEV/HI " " (today's)

1 µl DNA mass ladder
50
60
20
10
5
2.5

Ligations set up:

	14.1.98	2.2.98
vector	2.5 µl	2.5 µl
insert	4.5	4.5
ligase	1.0	1.0
H_2O	5.5	6.5

inc. RT O/N on bench.

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Haray	M. Graham
Signed	Signed	Signed
2/2 98	2/2/98	10.2.98
Dated	Dated	Dated

Experiment

E coli Transformation

Date 4/2/98

Continued From Page #: 26 Book #: 32

Aim: to transform 2 ligations from p.26 into DH5α chemically competent cells prepared yesterday.

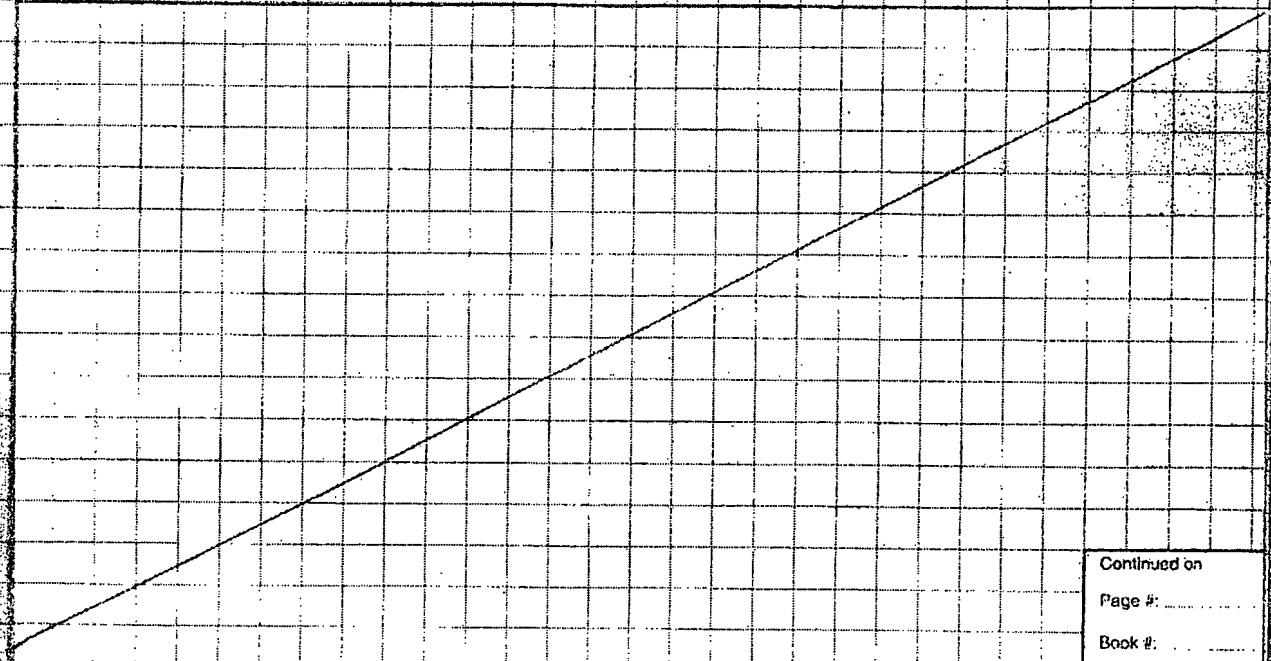
Method:

100µl DH5α

①	lign 1 (14.1.98)	4µl
②	lign 2 (2.2.98)	4µl
③	1pg pUC18	1µl of 1pg/µl dilution (used 1µl from pUC18 stock 10ng/µl to prepare dilutions)
④	50pg pUC18	1µl of 50pg/µl dilution
	-ve control	

ice, 20-30', heat shock 42°C/90", recovery in 900µl SOC plated out 100-200µl onto kan100µg/ml or Amp 100µg/ml plates.

inc. O/N @ 37°C



Continued on Page #: Book #:

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	V. Hardy	M. Graham
4/2/98 Dated	VHardy Signed	4/2/98 Dated
		10/2/98 Dated

Title of Experiment: Expt - 7

Transformation results.

Date: 5/2/98

Continued From Page #: 27 Book #: 32

to calculate transformation efficiency to new prepared DH5α cells:

370 colonies / 100µl plated out from 50pg transformation
= 5pg DNA x 200 = ng DNA
x 1000 = µg DNA
7.4×10^6 CFU / µg DNA
= 7.4×10^7 CFU / µg DNA

Reasonable efficiency with new cells. Checked on kan and amp plates for resistance, no colonies observed after O/N inc @ 37°C.

Result of transformation with ligation ① & ② = pEGFP-N1 + BEV.

Lign ① 100µl & 200µl plates
colonies TNTC
colonies selected for counterselection on both kan + amp plates.

② same as above.

Expect alot of religated vectors:

either pCR2.1/BglII+BamHI amp^R/kan^R
pEGFP-N1/BglII+BamHI kan^R
} both present in each ligation

on counterselection plates looking for kan^R colonies, as these should be the pEGFP-N1 vect

Continued on Page #: 29 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
M Bernard 5/2/98	V Hardy 5/2/98	
Signed / Dated	Signed / Dated	Signed

Page 29

Date 6/2/98

Continued From Page #: 28 Book #: 32

Counterselection results + PCR screening of putative transformants

Counterselection results

37 kan^R colonies from ligation 1

66 kan^R colonies from ligation 2

PCR screening:

Method: one colony per 10µl LB, toothpick shaken in LB briefly + discarded. LB tubes placed at 37°C while setting up PCR master mix. Vortex + briefly spin tubes before adding broth to master mix

BEV master mix

		× 25
H_2O	9.9µl	247.5µl
10mM dNTP's	0.25	6.25
10µM BEV-1	0.25	6.25
" BEV-2	0.25	6.25
10×PCR buffer	1.25	31.25
BM Taq	0.1µl	2.5
broth	0.50	–
	12.5	300.0 → 12µl per tube + 0.5µl broth + colony.

Program #48 94 → 47 → 72 → 15

#47

94°C 30" } ×35 cycles
60°C 1' }
72°C 1' }
68°C 5'
15°C ∞

gel loading buffer added to samples on run on 1% TAE agarose gel. 90V | 35'.

Continued on Page #: 30 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	V. Mardy	M. Graham
6/2/98 Dated	V Mardy Signed	6/2/98 Dated
		19 2 98 Dated

Title of Experiment: Expt. 6 cont.

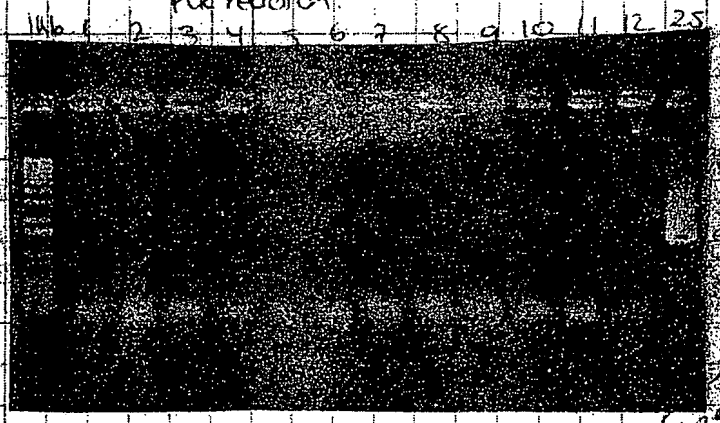
PCR results / colony screening of pECFP+BEV putative transformants

Date: 6/2/98

Continued From Page #: 29 Book #: 32

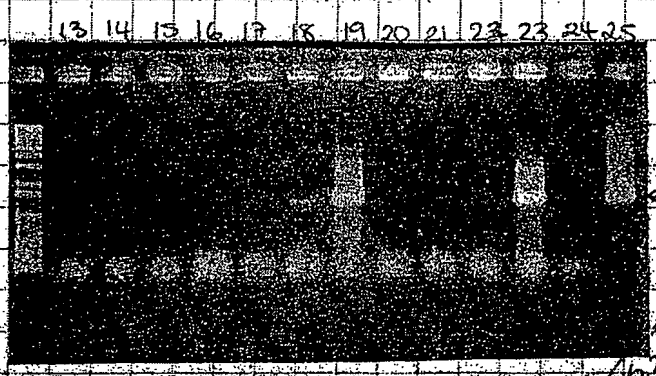
1-22 colonies 1-22
23 $Kan^R Amp^R$ colony
24 broth control
25 PLR2-1+BEV#1 0.5 µl plasmid DNA

lane	
1	0.4µg 1Kb
2	1
3	2
4	3
5	4
6	5
7	6
8	7
9	8
10	9
11	10
12	11
13	12
14	½ of 25



faint bands with rxns 1, 3, 8, 10

lane	
1	0.4µg 1Kb
2	13
3	14
4	15
5	16
6	17
7	18
8	19
9	20
10	21
11	22
12	23
13	24
14	½ of 25



faint bands with rxns 17, 18, 20, 22
strong band with rxn 19
rxn 23 $Kan^R Amp^R$ colony could be PLR2 1+BEV
24 -ve broth control ok
25 +ve control ok

Continued on Page #: 31 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MQB (Signed)	6/2/98 (Dated)	VHardy (Signed) 6/2/98 (Dated)

Expt. 6 cont.

Minipreps of putative transformants

Date 8/2/98

Continued From Page #: 30 Book #: 32

LB+kan 100mg/ml cultures set up O/N @ 37°C.
pEGFP+BEV

singles: #1, 3, 8, 10, 17, 18, 19

pools: kan^R colonies selected

		colony # on plate
ligation ①	☐1	23, 24, 25, 26, 27, 28, 36
	☐2	29, 30, 31, 32, 33, 34, 35

	pool #	
ligation ②	1	1+3 — 4+3 colonies
	2	5 colonies
	4	+9 — 4+3 colonies
	5	6 "
	6	6 "
	7	+14 — 4+3 "
	8	4 "
	10	4 "
	11	5 "
	12	5 "
	13	5 "
	16	5 "

grown ~23 hours @ 37°C.

1/BB 8/2/98

Qiagen minipreps, eluted in 100µl, digested 5–15µl with BamHI / BglII. Some cultures did not grow.

BEV pools

		x14
DNA	15.0	
10x buffer M	2.0	28
BglII	0.5	7
BamHI	0.2	7
H_2O	2.0	28
	20.0	70

→ dispensed into 5µl aliquots

BEV singles

		x4
DNA	6.0	
10x buffer M	2.0	8.0
BglII	0.5	2.0
BamHI	0.5	2.0
H_2O	12.0	48.0
		60.0

→ dispensed into 5µl aliquots

DNA added, inc. 2 hours @ 37°C.

PTO gel results of digests

Continued on Page #: 32 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
N. Bernard	V. Hardy	M. Graham
Signed	Signed	Signed
9/2/98 Dated	9/2/98 Dated	10/2/98 Dated

Page 32

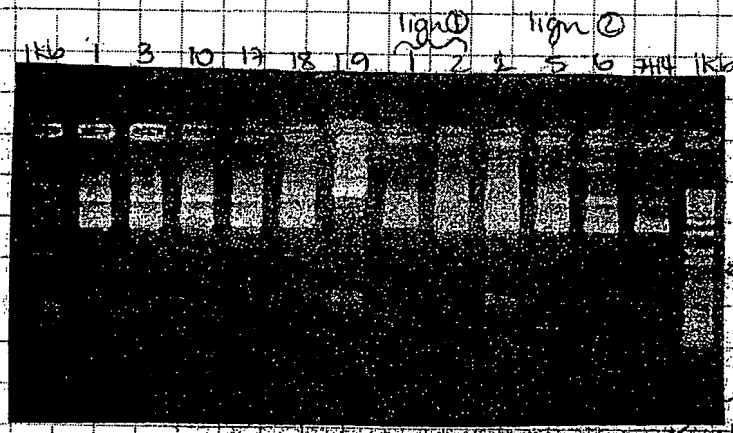
Title of Experiment: Expt. 6 cont.

Date: 9/2/98

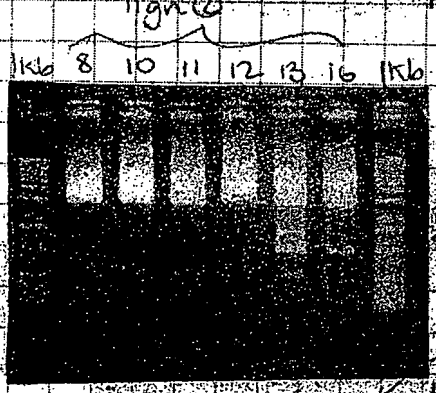
Continued From Page #: 31

Book #: 32

Digests results on miniprep:



NB note with most of mini's (except 13 of 6 from lign ②) were spun down before adding buffer N3, resuspended in 65µl N3 added to protocol cont.



Results: no 1.4Kb bands in top gel. Expected a band with #19 as there was a strong band with PCR (#19 could have been a Kan^R Amp^R colony?)
- 1.4Kb with pool #13 from ligation 2.

Next: Set up singles from pool #13 + repeat #19

Continued on Page #: ____ Book #: ____

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M Graham
MB Signed	9/2/98 Dated	Signed 9/2/98 Dated

Expt. 6 cont.

Page 33

Date 10/2 98

Continued From Page #: 32 Book #: 32

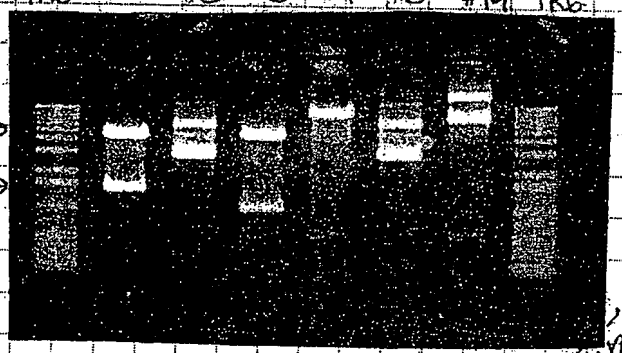
Minipreps on putative pEGFP-N1 +BEV transformants

Aim: to check single minipreps from pool 13 (p 32) and to repeat clone #19 (p 30)

Method: ①-⑤ = pool 13 (5 singles) ⑥ = repeat of #19
Qiagen miniprep spin columns on 2ml O/N cultures
eluted in 100µl MQ H_2O
digested 5µl DNA with BglII + BamHI

		x6
DNA	5.0	
BM 10X buffer(M)	2.0	12.0
BM BglII	0.5	3.0
" BamHI	0.5	3.0
H_2O	12.0	72.0
	20.0	90.0 → 15µl aliquot + DNA, inc. 37°C / 1.5hrs

RESULTS:



1% TAE gel
80V / 45'
stained in EtBr.

NP 10/2/98

- only one clone has a 1.4Kb fragment, expected to be the BEV polymerase → clone #1 from pool 13

NEXT:
- sequence clone #1 → check which primers to use.

Continued on Page #: 34 Book #: 32

Witnessing Experiment	First Witness of Experiment	Second Witness of Experiment
Read and Understood	V. Hardy	M. Graham
10/2 1998 Dated	V Hardy Signed	10/2 1998 Dated
		10/2 98 Dated

Page 34

Title of Experiment Expt. 8

Sequencing of pEGTPNI+BEV polymerase clone plus PCR2.1 clones + BEV. pol.

Date 11/2/98

Continued From Page #: Book #:

PCR2.1+BEV polymerase

(sequencing reactions set up on 28.1.98 using original FS mix)

		Reaction		Primer
8.0 µl	FS mix	1	PCR2.1+BEV clone #1	Univ.
0.3 µl	primer	2	"	Univ.
5.0 µl	miniprep DNA	3	PCR2.1+BEV clone #3	Univ.
6.7 µl	MQ H_2O	4		Univ.
20.0				

Program #67 (9600 PE machine)

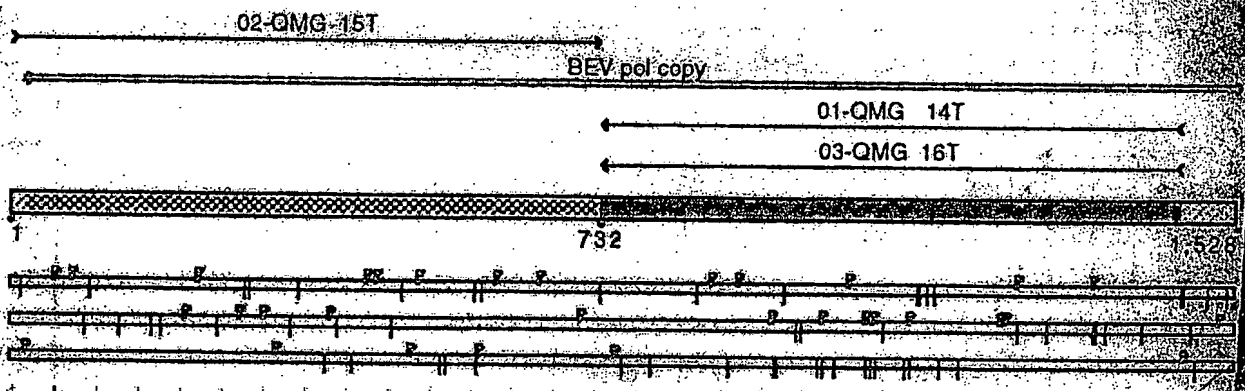
Cleaned up using 2 µl 3M Na acetate, pH 5.2 + 50 µl 100% ethanol, spin 14K/4°C for 20', 70% wash, dry pellet.

	Rxn
QMG 14	1
15	2
16	3
17	4

Results: sequences downloaded to Sequencher, aligned with original BEV polymerase sequence, contig formed

Contig [00061]

Sequencher™ "PCR2.1+BEV analysis"



QMG 17T did not align into contig: analysed individually

PCR2.1+BEV clone #1 mutation in sequence within primer BEV-1 (AA instead of AG)

PCR2.1+BEV clone #3 mutation in sequence within primer (AA instead of AAA)

PRIMER BEV-1S CGGCAGATCTAACAA TGGCAGGAC AAA ATCG AGTT (mutation)

Continued on Page #: 35 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
Signed: MBernard	Signed: VHardy	
Dated: 12/2/98	Dated: 12/2/98	

Mutation clone #3

Expt. 8 cont.

Sequencing cont. PCR2 HBEV pol

Date 11/2/98

Continued From Page #: 34 Book #: 32

Set up sequencing reactions: USING BIG DYE TERMINATOR MIX

PCR2 HBEV polymerase, clones #1, #3, #4

new primer designed to check for ambiguities from sequencing ~~of~~ results obtained with univ F + R primers (p. 34)

new primer is BEV-9, conc: 15 µg MW 7079.6

5' TTC TTG TGG AGG ACA GCC GGT TC 3'

$\frac{15\,\mu g/210\,\mu l}{7079.6} \times 1\times10^6 = 102\,\mu M$

diluted to 10 µM to set up sequencing, stocks stored @ -20°C.

DNA	4.0 µl (miniprep Qiagen spin column)
BigDye	8.0
10 µM primer	0.3
H_2O	7.7
	20.0

see p. 10 for gel photo of DNA digest with BamH/BglII

SEQ rxn		label	
1	PCR2 HBEV #1	OMG-23	Program 67 (9600 PE machine)
2	" " #3	" 24	
3	" " #4	" 25	

MJH 2 98

Cleaning up Seq rxns:

20 µl seq rxn + 2 µl 3M Na acetate + 52 µl 95-100% ethanol

↓

-20°C | ~20'

↓

14K | 4°C | 20' remove SN

↓

2x 70% ethanol washes

↓

pellets dried

Continued on Page #: 36 Book #: 32

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Teresa M	V. Hardy	M. Graham
Dated 12/2/98	Signed V Hardy Dated 12/2/98	Signed Dated 2/2/98

Title of Experiment EXP. 8 cont.

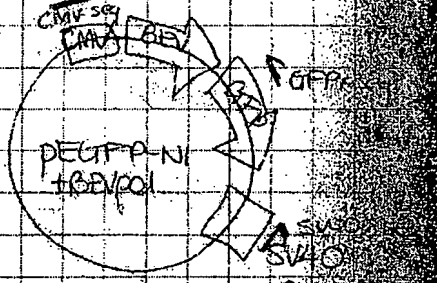
Sequencing cont. pEGFP+BEV clone #1

Date 11/2/98

Continued From Page #: 35 Book #: 32

Aim: to sequence pEGFP+BEV polymerase clone #1 from pool 3 (p.33) using oligos designed for pEGFP-N1 vector

location of primers:



PRIMERS (resuspended in MQ. H_2O):

SV40r. seq

132 µg / 210 µl MW 6188 mM = 102 µM

5' TTA TGT TTC AGG TTC AGG GG 3'

GFPr. seq

132 µg / 200 µl MW 6095 mM = 101 µM

5' CTG AAC TTG TGG CCG TTT AC 3'

CMV. seq

132 µg / 210 µl MW 6230 mM = 101 µM

5' CGG TGG GAG GTC TAT ATA AG 3'

SET UP SEQUENCING REACTIONS: All primers diluted to 0.3 µM

DNA	4.0 µl (Qiagen miniprep DNA) see p 33
10µM PRIMER	0.3
BIGDYE	8.0
H_2O	7.7
	20.0

Reactions run with others on p.35

clean up as on p. 35

label	SEQ. RXN		PRIMER
CMG26	4	pEGFP+BEV #1	SV40
CMG27	5		GFP → only 10µl as tube split when setting up reacts
CMG28	6		CMV

sequencing results analysed → confirms mutation in clone

Continued on Page #: 38 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MB Signed	12/2/98 Dated	V Hardy Signed
	12/2/98 Dated	

Expt. 9

PCR CLONING + OTHER CLONING

Date 17/2/98

Continued From Page #: Book #:

Cloning to be done soon:

construct	primers	template
PCR BEV 2	BEV-1 + BEV-3	pK277 #2
PCR BEV 3	BEV-4 + BEV-3	"
PCR. BamGFP Bgl II	GFP-Bam + Bgl GFP	pEGFP-N1 MCS

pEGFP-N1 MCS

pCMV. cass — N/A — pEGFP-N1 MCS PinAI / NotI Blunt end fill religate.

Primers resuspended in MQ H_2O

			STOCK CONC
GFP Bam	17 µg / 210 µl	MW 7893.2	103 µM
Bgl-GFP	17 µg / 215 µl	MW 7983.2	100 µM
BEV-3	108 µg / 120 µl	MW 8928.8	100 µM
BEV-4	105 µg / 125 µl	MW 8600.6	98 µM

PCR not set up, as decided to set up sequencing of PCR2.1+BEV #4 (universal forward / reverse primers) as results of sequencing analysed today for clones #1 + 3. see p. 38 for details.

Continued on Page #: 46 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
[illegible]rnard	V. Hardy	M. Gwenham
[illegible]rnard 17/2/98 Dated	V. Hardy 17/2/98 Signed / Dated	25/2/98 Dated

Page 38

Title of Experiment: Expt. 8 cont.

SEQUENCING : PCR2.1 + BEV clone #4

Date 17/2/98

Continued From Page #: 36 Book #: 32

Aim: to sequence clone #4 as results of sequencing with BEV-S primer have confirmed mutations in clones #1 + #3 (see p. 39)

Method

BIG DYE	8.0 µl	
DNA	4.0	miniprep, Qiagen spin
10µM primer	0.3	
H_2O	6.7	
	20.0	program 67
		cleaned up as on p. 35

Reaction	label	primer
1	OMG29	Universal forward
2	OMG30	Universal reverse

Continued on Page #: 43 Book #: 32

Name of Person Conducting Experiment: M. Bernard — Signed: MBernard 17/2/98 — Dated

First Witness of Experiment: V. Hardy — Signed: VHardy 17/2/98 — Dated

Second Witness of Experiment: [signature]

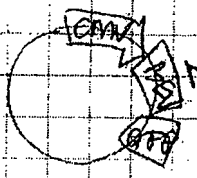
Expt. 8 cont.

Date 17/12/98

Continued From Page #: 35 Book #: 32

SEQUENCING RESULTS WITH BEV-5 PRIMER

Aim: to analyse results from sequencing of PCR2.1 +BEV clones #1, 3 and 4 with BEV-5 primer.



Primer designed approx 100bp within BEV polymerase (reverse direction)

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM

Page 1

Sequence Range: 1 to 1471 BglII

BEV-1 5' CGG CAG ATC TAA CAA TGG CA 3'

End of primer BEV-1

5' CCACCCAGAC TAAGCTAGAA CCCAGCGTTT TCTTCGACGT CTTCCCGGGT GTTAATGAAC
GUTGGGTCTG ATTCGATCTT GGGTCGCAAA AGAAGCTGCA GAAGGGCCCA CAATTACTTG
ProThrGlnThr LysLeuGlu ProSerVal PhePheAspVal PheProGly ValLysGlu>

BEV-5 PRIMER 3'

5' CGGCTGTCGT CCACAAGAAG GACAAGAGGC TTGAGACCAA CTTTGAAGAA GCCCTGTTTT
GCCGACAGCA GGTGTTCTTC CTGTTCTCCG AACTCTGGTT GAAACTTCTT CGGGACAAAA
ProAlaValLeu HisLysGlu AspLysArg LeuGluThrAsn PheGluGlu AlaLeuPhe>

18/2/98 µls overlapped from p.2 this book.

Sequences checked → go ahead with cloning using #4 µls 18/2/98

Results: comparison to reverse complement sequence (starting at end of BEV-primer in reverse direction)

PCR2.1 +BEV #1

expected: 5' ~~TGTCA TGCC GAT TGCC TGTC CATC TCC CTTC~~

actual ✓✓✓ (X) ✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

A→C mutation

confirms result from p.34

sequence is AA instead of ACA within primer region of BEV-1

PCR2.1+ BEV#3 (compared to above expected sequence)

actual ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ (X) ✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

T→G mutation

A→C A→C

confirms result from p.34

sequence is AA instead of AAA within primer region of BEV-1 (sequence highlighted above)

PCR2.1+ BEV #4 ~~is in opposite orientation... oops looking at reverse strand~~ universal PRIMER

actual: ~~universal seq~~ CCC GGG ATC CTC GAA AGA ATC GTA CCA CTT C ← BEV-2

Qualseq: ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

~~seq~~ used

no mutations with clone #4 ∴ check universal ✓✓ ~~and~~ forward + reverse sequences (set up on p.38) PRO p.40

Continued on Page #: Book #:

Witnessing Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	J. Hardy	M. Graham
Dated 23/12/98	Signed J. Hardy Dated 23/12/98	Signed Dated 23/12/98

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Title of Experiment EXPT. 98 cont

NEW CLONING: pEGFP + BEV (clone#4 of PCR2.1+BEV)

Date 18/2/98

Continued From

Page #:

Book #:

Aim: - to use PCR2.1 +BEV clone#4 to clone into pEGFP
- results from p.39 suggest clone#4 doesn't have any mutations in the BEV-1 primer region whereas clones #1 and #3 have mutations.

Method:

insert

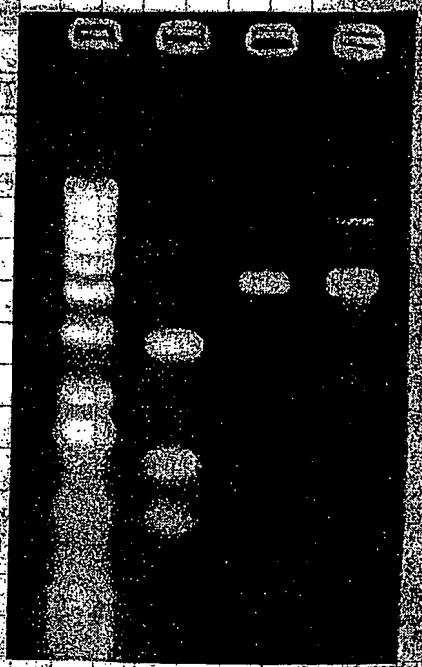
PCR2.1 +BEV#4 (13.1.98) Qiagen mini DNA		15.0 µl
BM 10x buffer M		5.0
BM Bgl II		1.0
" BamHI		1.0
H_2O		28.0
		50.0

inc 2 hrs @37°C

vector

pEGFP-N1 / Bgl II / BamHI
DNA remaining from 2.2.98 and also SAP treated DNA from 26.1.98

precipitation of insert DNA
5µl 3M Na acetate + 125µl 100% ethanol + 50µl digest
spin 20'/14K @4°C
70% ethanol wash, resuspended in 20µl H_2O
aliquot run on gel with vectors



1% TAE agarose gel
1 5µl 1kb loader
2 2µl PCR2.1+BEV#4 / BH1 / Bgl II
3 pEGFP / BH1 / Bgl II (2.2.98) 1µl
4 " " +SAP (26.1.98) 1µl
5 -

LIGATIONS

	-SAP	+SAP
pEGFP-N1 / Bgl II + BamHI	2.5µl	5.0µl
insert	4.0	4.0
10x lig buffer	1.0	1.0
ligase	1.0	1.0
H_2O	1.5	-
	10.0	11.1

inc 4°C O/N

Continued on

Page #:

Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	M. Graham
MMB	19/2/98	VHardy 19/2/98
Signed	Dated	Signed Dated

Expt. ~~8~~ cont.
6 cont.

TRANSFORMATIONS : pEGFP +BEV#4

Date 19/2/98

Continued From Page #: 40 Book #: 32

Aim:- to transform ligation from yesterday (p.40) into DH5α.

Method:-

electrocompetent DH5α, 50μl aliquots electroporated into DH5α (2002, 25μF, 2.5kV Biorad GenePulser)

(1)	pEGFP+BEV -SAP	3μl	43	kan
(2)	pEGFP+BEV +SAP	3μl	43	kan
(3)	pUC18 (50pg/μl)	1μl	4.6	Amp

Time constants.

Recovered in SOC, plated out onto kan or Amp plates

(1) 100μl + 200μl plated out, 250μl remaining
(2) 100 200 " " 550μl "
(3) 50μl " " 250μl "

Inc. 37°C/N → results: lots of colonies! control ok. 19/2/98

COUNTERSELECTION FOR KAN^R COLONIES

colony #
1-24 = pEGFP+BEV +SAP

25-90 = pEGFP+BEV

colonies streaked onto both kan + Amp plates, incubated on bench (2 days)

20/2/98

RESULTS FROM COUNTERSELECTION

colony #			all other colonies kan^R only ∴ 2-3ml LB + kan cultures set up for minipreps (pools)
	kan^R	Amp^R	pool (colony #) pool (colony #)
1-24			25-30 68-72
36	"	"	31-35 73-78 (-74)
38,39	"	"	37-42 (-38,39) 79-84 (-81)
48	"	"	43-47
51	"	"	49-54 (-51)
67	"	"	55-60
74	"	"	61-66
81	"	"	
89,90	"	"	

Continued on Page #: 42 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
C. Bernard	V. Hardy	M. Graham
C. Bernard 22/2/98 Dated	V. Hardy Signed	22/2/98 Dated
		Signed 25/2/98 Dated

Page 42

Title of Experiment Expt 8 cont 6

POOLED MINIPREPS : PUTATIVE pEGFP + BEV CLONES

Date 23/2/98

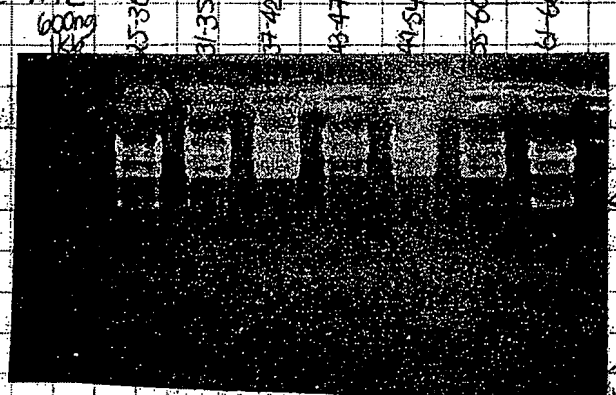
Continued From Page #: 41 Book #: 32

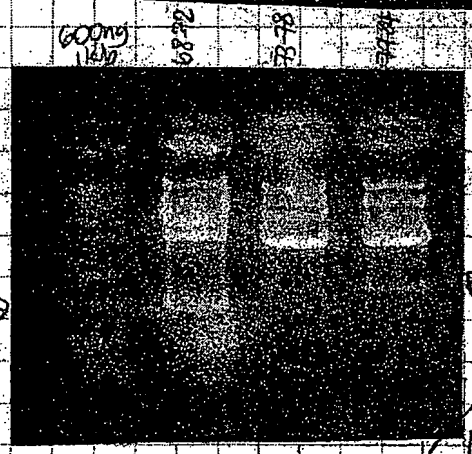
Qiagen minis, spin columns
eluted in 100µl MQ H_2O
digested 12.0µl with BamHI / BglII
x10

	DNA	12.0µl	
BM	10X Buffer M	2.0	20.0
"	BglII	0.5	5.0
"	BamHI	0.5	5.0
	H_2O	5.0	50.0
			80.0 → 8.0µl per tube + DNA

inc. 37°C / 1 hours

1% Agarose TAE





4 pools have a 1.1Kb band (BamHI/BglII fragment)

25-30 } Single
31-35 } Minis set up (2ml LB + Kan 100µg/ml)
61-66 } 25, 61, 62, 63, 64, 65, 66, 79, 80, 82
79-84 }

inc. 37°C O/N

Continued on Page #: 44 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Hardy	
M Bernard 23/2/98	V Hardy 23/2/98	
Signed Dated	Signed Dated	

SEQUENCING RESULTS : PCR2.1 + BEV clone #4

Date 23/2/98

Continued From Page #: 38 Book #: 32

Results:

PCR2.1 + BEV #4 FORWARD PRIMER OMG29
- signal quite low, only some readable sequence
- BEV-1 primer sequence located and checked → OK VV
- sequencing artefacts particularly in GGG regions.

PCR2.1 + BEV #4 REVERSE PRIMER OMG30
- signal a bit bigger, more readable sequence
- BEV-2 primer sequence located and checked → VV ok.
- some of BEV polymerase sequence checked → VV OK
- sequencing artefacts → T region ; GTG region, but peaks underneath are readable and obviously present.

Comments:

Cloning with PCR2.1 + BEV #4 should work!

Next: check single minis from pools, set up today.

Continued on Page #: Book #:

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	V. Hardy	M. Graham
23/2/98 Dated	23/2/98 Signed	23/2/98 Dated

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Title of Experiment Expt. 6 cont

MINIPREPS FROM POOLS : pEGFP + BEV

Date 24/2/98

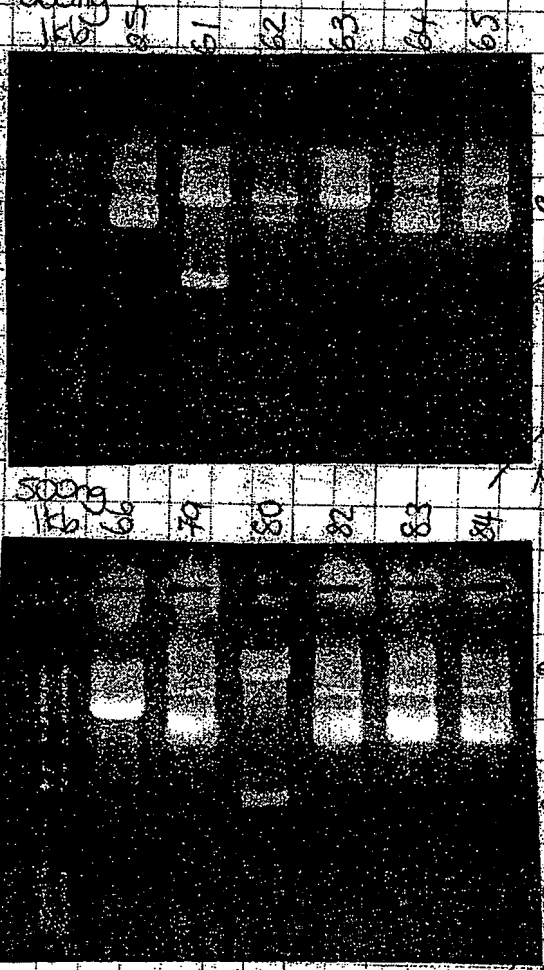
Continued From Page #: 42 Book #: 32

Qiagen minis, 1.5ml culture eluted in 100µl MQ H_2O digested 5.0µl with BamHI + BglII to release 1.4kb BER polymerase fragment.

		x12
DNA	5.0µl	
BM 10x (M)	2.0	24.0
" BglII	0.5	6.0
" BamHI	0.5	6.0
H_2O	12.0	144.0
	20.0	180µl → 15µl x12

+5µl DNA
inc. 37°C/1.5hrs

1% TAE agarose gel.



positive clones #61

↓

next: sequence this clone

see p. 45

#80?? vector is too large + smaller band present.

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	V. Harvey	
MBernard 24/2/98	VHarvey 24/2/98	
Signed Dated	Signed Dated	

EXPT. 10

SEQUENCING : pEGFP+BEV clone#61

Date 24/2/98

Continued From Page #: 44 Book #: 32

Aim: to sequence clone#61 with primers designed for pEGFP vector

Method used Big Dye Terminator mix

primers: as on p.36 10μM aliquots
SV40 r.seq → GFP sequence/SV40 junction
GFPr seq → look for GFP+BEV pol. (3'end)
CMV.seq → look for BEV polymerase (5'end)

Mini prep DNA (p.44) lane 3 of top gel, clone#61

		1 GFP	2 CMV	3 SV40
BIG DYE	8.0μl	✓	✓	✓
DNA	4.0	✓	✓	✓
10μM primer	0.3	✓	✓	✓
H_2O	7.7	✓	✓	✓
	20.0			

Method 67 (9600 PE)

Clean up
0.1vol Na acetate, 2.5vol 95% ethanol, -20°C/1 hour
14K/25' @4°C
2x 70% ethanol washes
pellets dried

OMGr 31 pEGFP+BEV clone#61 GFP.seq
" 32 " " CMV seq
" 33 " " SV40r.seq

Continued on Page #: 50 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M-Bernard	V. Hardy	W. Graham
Dated 24/2/98	Signed V.Hardy 24/2/98 Dated	Signed 3/3/98 Dated

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Title of Experiment EXPT

NEW CLONING : PCR.BEV2
PCR.BEV3
PCR.BamGFPBgl

Date 26/2/98

Continued From Page #: 37 Book #: 32

Aim. to set up PCR to amplify fragments for final set of constructs (as above)

Method:

Primers resuspended as on p.37
10μM stocks prepared (10μl of 100μM stock + 90μl H2O)

Reaction

DNA	0.1-1.0μl
10μM primer 1	0.25μl
2	0.25μl
BM 10x buffer +Mg	5.0μl (Lot C8300552O-22)
50mM dNTP's (Invitrogen)	0.5μl
BM Taq 5u/μl (3 Prep)	0.2μl
H2O	42.8μl

Final Construct Name	Reaction/Primers	DNA
	1 } ~~Cloning~~/PNV work	
	2 }	
PCR.BEV2 (Transgradies)	3 BEV1 + 3	1.0μl 1/10 diln. PE2774-2
PCR.BEV3 (Non-trans)	4 BEV 4 + 3	"
PCR.BamGFPBgl	5 GFPBam + Bgl-GFP	0.1μl pEGFP mini (1μg/μl) (3.1.98) gel on p.7
	6 } PNV work	

MB 6/3/98

THIS IS incorrect those names are of the clones in the PA cloning vector only MB 6/3/98

Method 26 (9600 PE Plant 26)

22		94°C	1:30
↓			
23		94°C	1:00
	↓	55°C	1:00
	↓	72°C	1:30
24		72°C	10:00
↓			
15		15°C	∞

Continued on Page #: 47 Book #: 32

Name of Person Conducting Experiment: M. Bernard

First Witness of Experiment: V. Hardy

Second Witness of Experiment: M. Gardner

M Bernard 26/2/98 Signed Dated

V Hardy 26/2/98 Signed Dated

Expt. 9 cont.

PCR results : new cloning

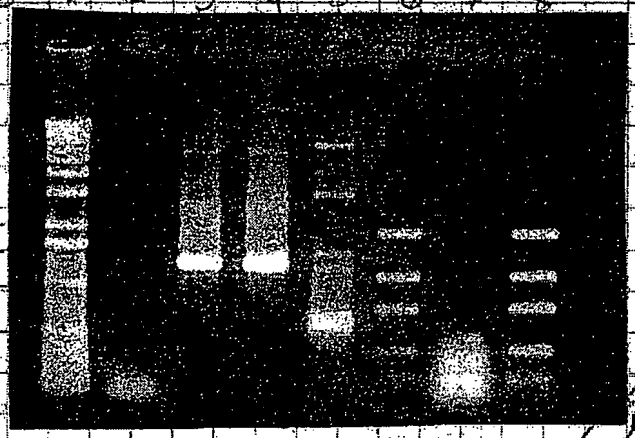
Date 27/2/98

Continued From Page #: 46 Book #: 32

PCR reactions from p. 46
1% TAE gels

lane
1 1 µg 1Kb ladder (Gibco)
2 10µl reaction 2 = PWV WORK!
3 10µl " 3
4 " 4
5 " 5
6 1 µl DNA mass ladder (Gibco)
7 10µl PCR rxn 4 PWV (20.2.98)
8 2µl DNA mass ladder

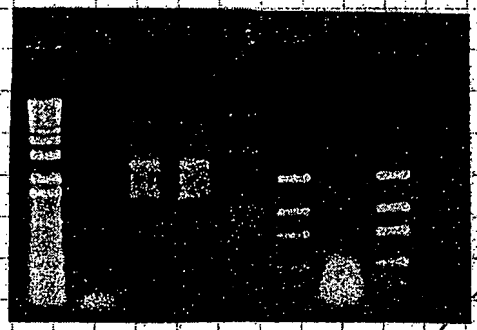
80V



mass ladder	
1µl ng DNA	2µl ng DNA
50	100
30	60
20	40
10	20
5	10

Uppalt

>100ng of each PCR product obtained (in 50µl aliquot)



bands from lanes 3, 4 & 5 cut out for gel purification

Uppalt

Continued on Page #: 48 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
C. Bernard	V. Hardy	
CBernard 27/2/98	VHardy 27/2/98	3/3/98
Signed Dated	Signed Dated	Signed Dated

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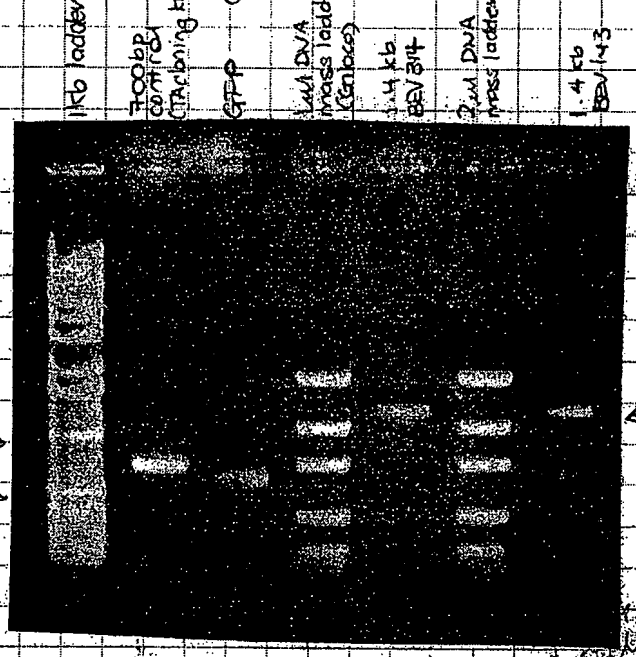
Date 2/3/98

Continued From Page #: 47 Book #: 32

Title of Experiment EXP1.1

Gel purification of PCR products.

Gel purification using Qiagen spin columns:
700bp control band (TA cloning kit) 1.39g - 1.02g = 0.37g
~700bp GFP band (Bam GFP + GFP Bgl product) 1.14g - 1.03g = 0.11g
1.4kb BEV band (BEV3+4 product) 1.12g - 1.025g = 0.095g
1.4kb BEV band (BEV1+3 product) 1.12g - 1.026g = 0.094g
3x buffer QG, all eluted in 30µl H_2O, speedivac to 1/2 vol
1µl aliquot run on gel.



DNA mass ladder	
1µl	2µl
50	100
30	60
20	40
10	20
5	10
	5
	2.5
	1

MMB 2/3/98

SET UP LIGATIONS INTO PCR2.1 (TA CLONING KIT-INVITROGEN)
approx. conc. from gel:
① GFP 5-10ng/µl
② BEV3+4 5-10ng/µl
③ BEV1+3 5-10ng/µl
④ 700bp control 2ng/µl

		control
PCR2.1	2.0µl	2.0µl
10x buffer	1.0	1.0
insert	3.0	2.0
ligase	1.0	1.0
H_2O	3.0	4.0
	10.0	10.0

INC. 14°C O/N

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M.M. Bernard	P. Campbell	M. Cashman
MMB	2/3/98	02/03/98
Signed	Dated	Signed Dated

EXPT. 11 cont.

Date 3/3/98

Continued From Page #: 48 Book #: 32

Transformations: PCR2.1 ligations (GFP, BEV143, BEV344 TA cloning kit control)

Aim: to use kit cells (TOP10F') heat shock protocol

Method: as per TA cloning kit instructions.

3µl of each ligation

plated 100µl + 200µl onto LB+Amp100 (actually <200µl)

inc. 37°C O/N

MB 3/3/98

Results:

# colonies		blue	white	
700bp control		89	2	→ low. no. of white colonies
GFP	100µl	80	>100	→ 39 colonies picked 12 screened
	~200µl	>100	>100	
BEV143	100µl	>100	>100	→ 42 colonies picked #1-42
	200µl	>100	>100	
BEV344	100µl	>100 (some light blue)	~30	→ 42 colonies picked #43-84
	200µl	>100	>30	

NEXT: PCR screening of white colonies.

Continued on Page #: 51 Book #: 32

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P. Campbell	M. Cursham
Bernard 4/3/98	Campbell 4/03/98	M. Cursham 4/3/98
Dated	Signed Dated	Signed Dated

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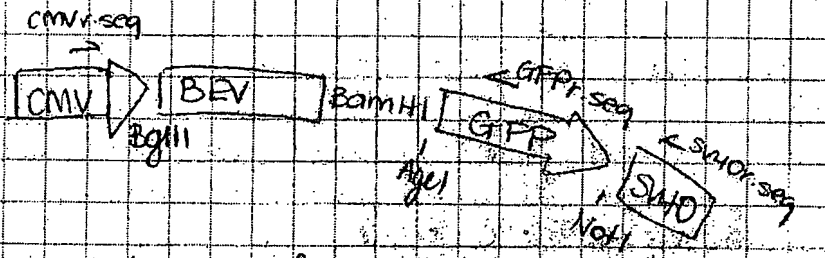
Title of Experiment EXPT. 10 CONT.

Date 4/3/98

SEQUENCING RESULTS: PEGFP + BEV clone #61
(=POR.BEV.1) PEGFP.BEV.1

Continued From:
Page #: 49
Book #: 32

sequencing results for: OMG 31 GFPr.seq
OMG 32 CMV.seq
OMG 33 SV40r.seq



downloaded seq from hot shots to sequencher looked for BamHI or BglII sites or NotI site, depending on primer.
(no printouts from AGRF)

GFPr.seq result:

vector sequence - MCS of pEGFP - BEV-2 primer - BEV polymerase sequence
BamHI site ✓
sequence edited and checked → ok ✓

CMV seq result:

vector sequence - BEV-1 primer - BEV polymerase sequence edited and checked → ok ✓
BglII site ✓

SV40r.seq result:

located NotI site, have not checked 3' end of GFP seq

Next:
prepare more DNA of clone #61 for transfections
glycerol stock prepared on 6/3

Continued on
Page #:
Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	P. Campbell	M. Gilchrist
MBernard 4/3/98	Campbell 04/03/98	
Signed / Dated	Signed / Dated	

EXPT. 11 cont.

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PCR screening · PCR2.1 + GFP
" BEV143
" BEV344

Date 5/3/98

Continued From Page # 49 Book # 32

Aim: to screen 12 white colonies of each of above transformations

Method:

PCR master mix

		x 39	
10μM Forward	0.2μl	7.8μl	
10μM Reverse	0.2	7.8	
10x PCR buffer + Mg	2.0	78.0	(lot 83005520-22)
Taq (31 Jan 99)	0.2	7.8	
50mM dNTP's (Invitrogen stock)	0.1	3.9	
H_2O	17.3	674.7	
		780 → 20μl per tube	

colonies picked (yellow tip) → streaked onto fresh Amp plate → tip touched into PCR mix.

Program #26 (9600 PE machine)

94°C 1:30
94°C 0:30
55°C 0:45 } x35
72°C 1:00
72°C 5:00
15°C ∞

PCR reactions

1-13 20μl plate GFP colonies #1-12 #13 = blue

14-26 " BEV143 colonies #14-25 #26 = blue

27-39 " BEV344 colonies #27-38 ~~#39 = blue~~ #39 = blue

15μl of each PCR reaction run on 1% TAE gel (except #39 - not enough wells)

Continued on Page # 52 Book # 32

Name of Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P. Campbell	M Graham
Bernard 5/3/98 Dated	Signed 05/03/98 Dated	Signed 5/3/98 Dated

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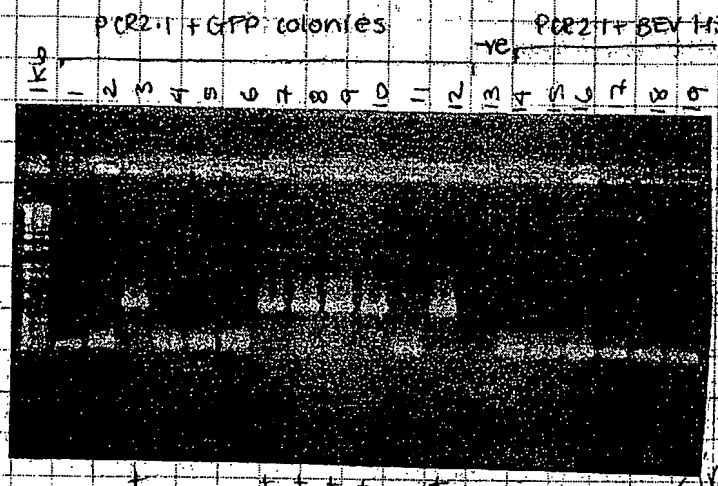
Title of Experiment EXPT. 11 cont.

PCR screening results : GFP
BEV 1-13
BEV 3-14

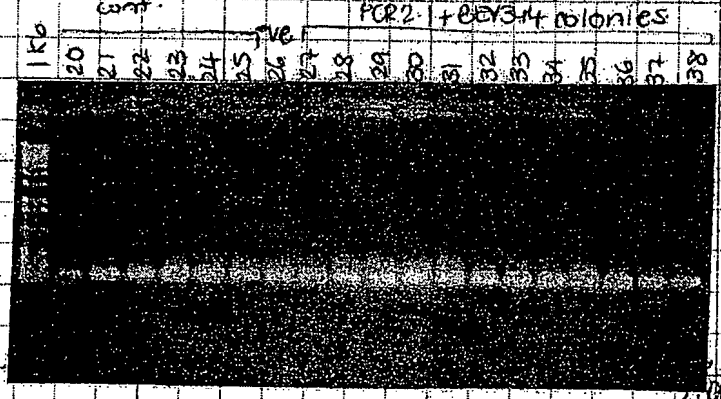
Date 6/3/98

Continued From Page #: 51 Book #: 32

1% TAE agarose gels



JMB 6/3/98



JMB 6/3/98

Comments:

6/12 positive for PCR2.1 + GFP → set up minis

0/12 " " PCR2.1 + BEV1-13 → set up pooled minis

0/12 " " PCR2.1 + BEV3-14 → set up pooled minis

LB + Amp cultures inc. O/N @ 37°C

Minis set up of 3, 7, 8, 9, 10, 12 (GFP)

Pooled minis 7 x 6 colonies of BEV1-13, BEV3-14 (1-42 colonies streaked onto numbered plate) BEV #1-42 (1-13), BEV #43-84 (3-14)

Continued on Page #: 53 Book #: 32

Name of Person Conducting Experiment: M Bernard — Signed: M Bernard — Dated: 6/3/98

First Witness of Experiment: P. Campbell — Signed: [signature] — Dated: 05/03/98

Second Witness of Experiment: M. Graham — Signed: [signature] — Dated: [illegible]

Expt. 11 cont.

Date 6/3/98

Continued From Page #: 52 Book #: 32

MINIPREPS : SINGLES OF PCR2.1 + GFP
POOLS OF PCR2.1 + BEV1+3
" " " + BEV3+4

Aim: to prepare mini-DNA for digests of above.
Method: Qiagen spin columns
1.5ml culture
eluted in 100µl H_2O
set up digests

POOLS x14 (7 pools PCR2.1 + BEV1+3
7 pools PCR2.1 + BEV3+4)
see below for colony #

		x14
DNA	12.0µl	
10x buffer H	2.0	28.0
BM ECOR1 10U/µl	1.0	14.0
H_2O	5.0	70.0
	20.0	112.0

larger aliquot of ECOR1 used exp. 31 Mar 98

		x7
GFP x6		
DNA	5.0µl	
10x buffer H	2.0	14.0
ECOR1	1.0	7.0
H_2O	12.0	84.0
	20.0	105.0

→ 12.0µl per tube instead of 15.0µl
clones # 3, 7, 8, 9, 10, 12.

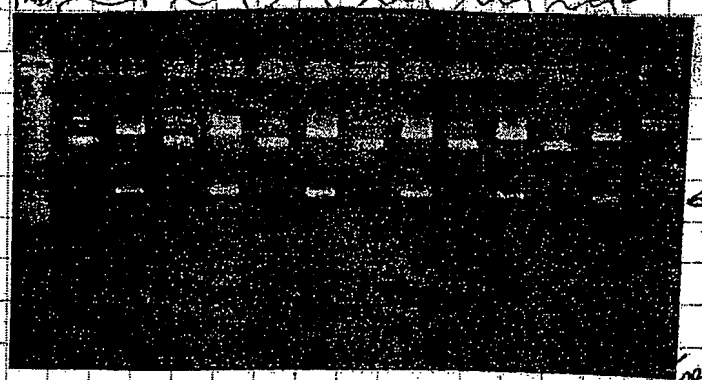
pools identity

	BEV1+3	BEV3+4
①	1-6	43-48
②	7-12	49-54
③	13-18	55-60
④	19-24	61-66
⑤	25-30	67-72
⑥	31-36	73-78
⑦	37-42	79-84

15 hours @ 37°C

15µl of digest loaded onto 1% TAE agarose gels.

PCR2.1 + GFP ~~clones~~ minis digested with ECOR1 (first lane uncut DNA, second lane ECOR1 digest)



← insert b/w 500 – 1000 kp

correct sized inserts with all minis
next: sequence → see p. 60
PTO for pooled minis / digests

Continued on: Page #: 54 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P. Campbell	M. Graham
Witnessed 6/3/1998 Dated	06/03/98 Signed Dated	4.3.98 Signed Dated

Page 54

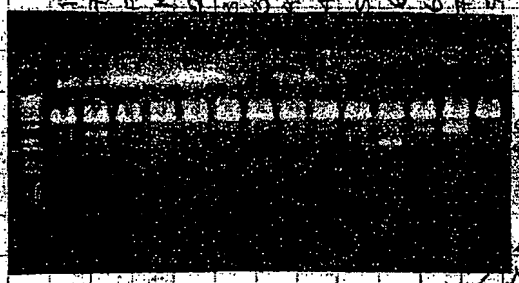
Title of Experiment: EXPT. 11 cont

MINIPREP DIGESTS CONT: PCR2.1 + BEV POOLS.

Date: 6/3/98

Continued From Page #: 53 Book #: 32

EcoRI digests of PCR2.1 +BEV pools
1% TAE agarose gel
90V/35', stained in EtBr.



PCR2.1+BEV1+3 → only one pool (7-12) is showing insert band, but it is too large.

PCR2.1+BEV3+4 → pool 61-66 has an insert band ~1.4Kb (could be larger?)

NEXT: set up single mini cultures from pool 61-66
screen more colonies from PCR2.1+BEV1+3.

Continued on Page #: 56 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	P. Campbell	M. Crea
MJB Signed	6/3/98 Dated	Signed 06/03/98 Dated

Experiment EXPT. 12

Page 55

Date 9/3/98

Continued From Page #: Book #:

MIDIPREPS: - PEGFP +BEV clone#61
= ~~PCR BEV.1 in patent~~ PEGFP.BEV.1 in patent appln
- PEGFP-N1 MCS

MB 2/3/98

Aim: to prepare larger amounts of DNA for mammalian cell transfections.

Method:

starter cultures: incubated 37°C ~6 hours

PCR.BEV.1 (clone#61)
-culture ~~from~~ for ~~miniprep~~ glycerol still in fridge (6/3)
4ml LB+kan50 + 0.5ml culture from fridge

PEGFP-N1-MCS
-glycerol stock (250µl) + 2ml LB+kan50

25ml cultures: used starter culture to inoculate following:

2x25ml (set up 75ml LB+kan) clone#61

1x 25ml (set up 50ml LB+kan) pEGFP-N1 MCS

37°C O/N

MB 9/3/98

Qiagen midi prep kit

PEGFP+BEV clone#61 (=PCR.BEV1) → 2x30ml } cells harvested in oakridge tubes 8K/5' @4°C
PEGFP-N1-MCS → 2x25ml }

↓
kit protocol followed
↓
DNA ppt'd in Corex tubes (2 tubes per DNA)
10K/30' @4°C
↓
70% wash ethanol (2ml/tube)
↓
pellet air dried 20'
↓
resuspended in 100µl MQ.H_2O.
stored @4°C O/N

Continued on Page #: 58 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P Campbell	M. Graham
Bernard 10/3/98	Campbell 10/03/98	2/3/98
Signed / Dated	Signed / Dated	Signed / Dated

Page 56

Title of Experiment EXPT. 1 cont.

PCR2.1+BEV1+3 → screening of more colonies for pos
" BEV3+4 → minis #61-66 = PCR.BEV.3 w/ pos

Date 9/3/98

Continued From Page #: 54 Book #: 32

Aim: to set up miniprep cultures of above

Method:

minis 61-66 = PCR2.1+BEV3+4 singles
LB+kan 1-2ml cultures.

pools 1-6 = PCR2.1+BEV1+3 white colonies
7-12 " "
13-18 " "
19-24 " white+blue colonies
LB+kan 1-2ml cultures.

37°C o/n

10/3/98

Qiagen minispin columns
1.5mls culture
eluted in 100µl MQ H_2O

Miniprep digests

singles (61-66) — NB #62 didn't grow

		x7
DNA	5.0µl	—
BM 10x buffer H	2.0	14.0
BM EcoRI (10u/µl)	1.0	7.0
H_2O	12.0	84.0
	20.0	105.0

larger aliquot → 7x15µl

pools (x4)

DNA	12.0µl
BM 10x buffer H	2.0
BM EcoRI (10u/µl)	1.0
H_2O	5.0
	20.0

inc. 37°C/2 hrs.

15µl loaded onto 1% TAE agarose gel.

31 Mar 98

Continued on Page #: 57 Book #: 32

Name of Person Conducting Experiment: M. Bernard — Signed: MBernard 10/3/98 — Dated

First Witness of Experiment: P. Campbell — Signed: Campbell 10/03/98 — Dated

Second Witness of Experiment: M. Graham

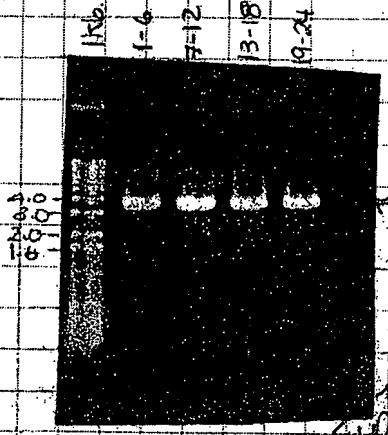
Expt. 11 cont.

Digest results: ① PCR2.1 + BEV 1-3 pools (= PCR.BEV.2 translatable BEV construct)

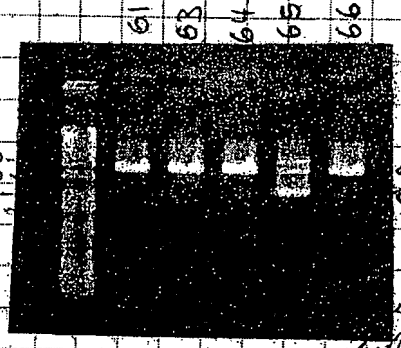
② PCR2.1 + BEV 3+4 clones #61-66 (= PCR.BEV.3 non-translatable BEV construct)

nB. names of final constructs in brackets ie when in expression vector pEGFP-N1-MCS

PCR.BEV.2
PCR2.1 + BEV 1-3 pools



PCR. BEV.3
PCR2.1 + BEV 3+4
clones # 61-66



Comments:

PCR2.1 + BEV 1-3 pools : - no insert bands in this batch of 24 colonies

PCR2.1 + BEV 3+4 clones 61-66: - insert band only with clone #65 but it is too large. Size looks greater than 2kb - a bit strange as band on previous gel (pool 61-66) looks < 2.0kb
- digests look a bit strange! could be due to old enzyme (exp. 3 March 98)

Next: - set up ligations again
- firstly clean up PCR products (use gel purification columns)
- Maybe repeat digests with a new EcoRI stock?

Continued on Page #: Book #:

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
S Bernard	P. Campbell	M. Graham
S Bernard 10/3/98 Dated	P. Campbell 10/10/98 Signed / Dated	M. Graham 24/3/98 Signed / Dated

Page 58

Title of Experiment: EXPT. 12 cont.

MIDIPREP DIGESTS: ~~PER.BEV.1~~ and PEGFP-N1-MCS
PEGFP.BEV.1

Date 11/3/98

Continued From Page #: 55 Book #: 32

Aim: - to check midiprep DNA by digesting with BamHI and/or BglII
- to determine concentration

Method:

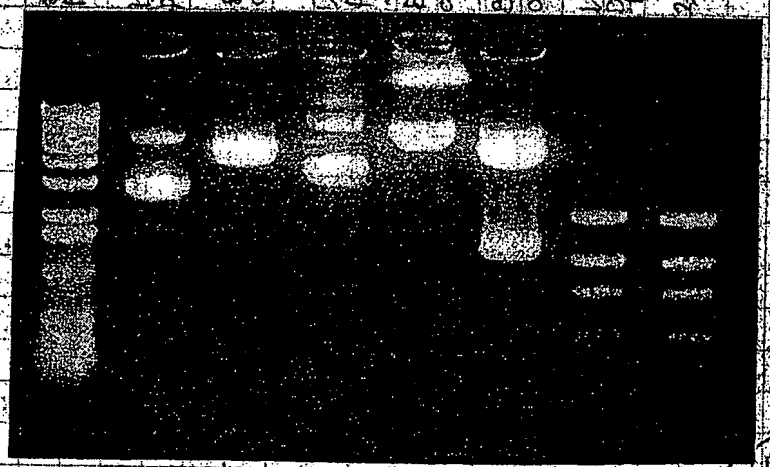
DNA from 4°C, 2x100µl aliquots combined & rinsed with an extra 100µl MQ H_2O (final vol ~300µl)

Digests:

DNA	2.0µl	2.0µl	2.0µl
10x buffer M	2.0	2.0	2.0
BamHI	1.0	1.0	1.0
BglII	—	—	1.0
H_2O	15.0	5.0	14.0
	20.0	20.0	20.0

inc 37°C 2 hours

2µl digest run on 1% TAE gel along with 1.0µl undigested DNA.



PEGFP-N1-MCS = 4.2kb, shown by BamHI digest

PEGFP.BEV.1

~~PER.BEV.1~~ = 3.6kb, shown by BamHI digest

BEV.1 insert = 1.4kb, shown by BamHI/BglII digest

concentration of both midipreps >100ng/µl

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	P. Campbell	M. Gibson
M Bernard 11/3/98	Campbell 11/03/98	24/3/98
Signed / Dated	Signed / Dated	Signed / Dated

EXPT. 12.Cont.

MIDIPREP CONCENTRATIONS

Date 11/3/98

Continued From Page #: 58 Book #: 32

spectrophotometry results:

1:60 dilution in NaH_2O.

11/3/98

1:60 dilution ng/ml

TUBE	A320	A280	A260	280/260	260/280	PROTEIN	NUCLEIC ACID
~~PCR-BEV.1~~ PEGFP-NI-MCS	0.0041	0.1990	0.3297	0.5985	1.6709	55.945	13.463
~~PCR-BEV.1~~ PEGFP-BEV.1	0.0085	0.3015	0.5408	0.5805	1.8167	51.642	22.976

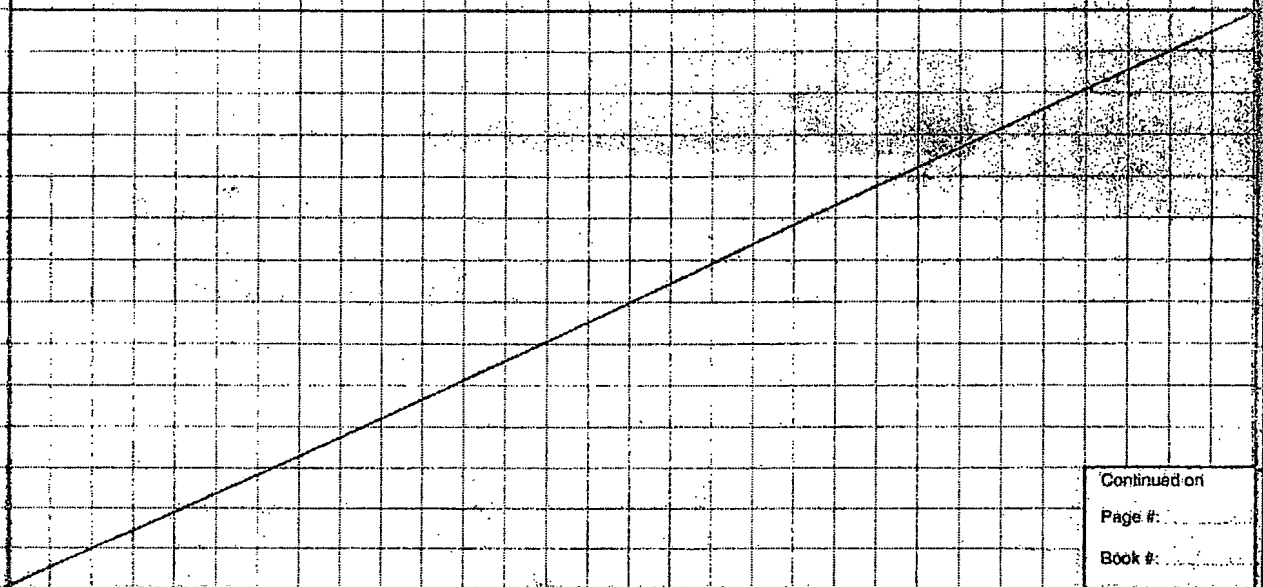
pEGFP-NI-MCS = 808 ng/ml
= 0.8 µg/ml

~~PCR-BEV.1~~ = 1376 ng/ml
PEGFP-BEV.1 = 1.4 µg/ml.

JM 20/3/98

MP 11/3/98

Mick has used the DNA to transfect cells today.



Continued on Page #: Book #:

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P. Campbell	M. Graham
11/3/98 Dated	11/3/98 Signed	11/05/98 Dated
Signed	Dated	24/3/98 Dated

Page 60

Title of Experiment EXPT. 13.

SEQUENCING: PCR2.1 + GFP done#3

(= PCR. Bgl - GFP - Bam).

Date 11/3/98

Continued From Page #: 53 Book #: 32

Aim: to check above clone by sequencing with universal forward + reverse

Method: sequencing reactions set up on 10.3.98
DNA of clone #3 digest shown on p.03
100ul miniprep DNA

DNA	4.0 ul
Big Dye	8.0
1uM primer	3.0
H_2O	5.0
	20.0

Program 67 (9600 PE machine)

↓

Clean up: as per p.45
only 1x 70% ethanol wash

QMG 36 F
QMG 37 R

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	P. Campbell	M. Graham
M Bernard 11/3/98	Campbell 11/05/98	
Signed Dated	Signed Dated	

EXPT. 11 cont

Page 61

Repeat of ligations: PCR2.1 + BEV1+3 (PCR.BEV.2)
PCR2.1 + BEV3+4 (PCR.BEV.3)

Date 12/3/98

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Aim: - to set up new ligations using cleaned up PCR product
- use PCR products → 50µl reactions originally set up on p.46 & only 10µl run on gel, which was gel purified for first ligation (see p.48)
- should be ~40µl remaining stored @ 4°C.

Rxn 3 BEV1+3
4 BEV3+4

- could be a problem if not gel purified as there is multiple bands (top gel photo on p.49) lanes 3+4

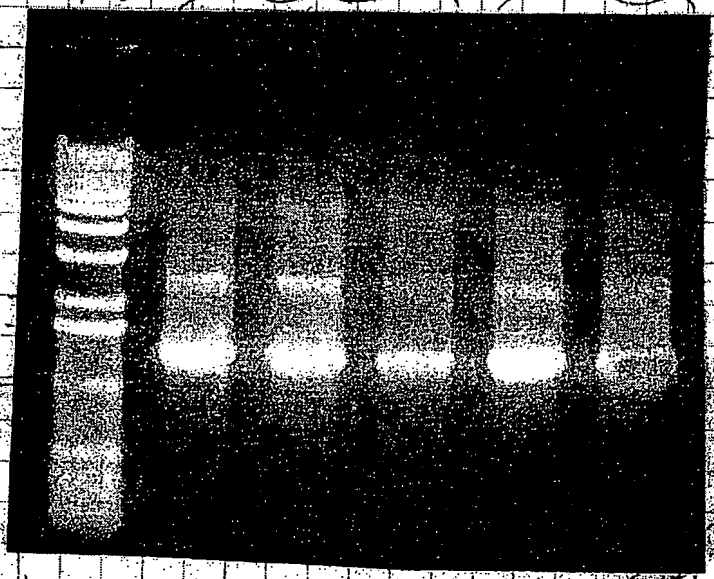
Method:

Remainder of reactions 3+4 run on 1% TAE agarose gel

~2ug 1Kb

PCR reaction (3) = BEV1+3

PCR Reaction (4) = BEV3+4



← 1.4Kb BEV PCR products bands cut out & combined for gel purification

photo after excising bands.

← 1.4Kb BEV products removed

Continued on Page #: 62 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
P. Bernard	P. Campbell	M. Graham
Bernard 12/3/98	12/03/98	2/3/98
Signed / Dated	Signed / Dated	Signed / Dated

Page 62

Title of Experiment: Expt. 11 cont.

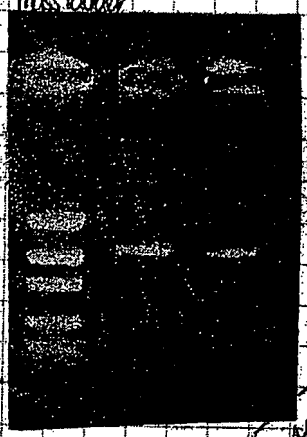
LIGATIONS CONT.: PCR2.1 + BEV1+3 (1.4kb) = (PCR.BEV.2)
PCR2.1 + BEV3+4 (1.4kb) = (PCR.BEV.3)

Date: 12/3/98

Continued From Page #: 61 Book #: 32

Gel purification of 1.4kb BEV PCR products:
using QIAquick columns
BEV1+3 = ② Wt. of bands ~0.3g x 3x buffer QG + 0.3ml isopropanol, eluted in 30ul
BEV3+4 = ④ wt. of bands ~0.25g x 3x buffer QG + 0.25ml isopropanol, eluted in 30ul

1ul aliquots of gel purified products run on 1% TBE gel.



WMB 12/3/98

Used Invitrogen TA cloning kit

PCR2.1 vector	2.0ul
10x buffer	1.5
insert	7.5
H_2O	3.0
ligase	1.0
	15.0

Inc. 14°C O/N

MB 13/3/98

13/3/98 Transformations into TOP10F' cells (from Invitrogen)

5ul each ligation

① PCR2.1 + BEV1+3
② PCR2.1 + BEV3+4

Heat shock protocol, recovery in 250ul SOC (from Invitrogen) Plated out 50 + 100ul onto LB+Amp+Xgal plates

Continued on Page #: 63 Book #: 32

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Bernard	P. Campbell	N. Gutierrez
W.O'Bernard 13/3/98	Campbell 13/03/98	
Signed / Dated	Signed / Dated	

Expt. 11 cont.

Page 63

PCR screening : transformations PCR2.1 + BEV.1+3 (=PCR BEV.2)
PCR2.1 + BEV 3+4 (=PCR BEV 3)

Date 16/3/98

Continued From Page #: 62 Book #: 32

Aim: to screen colonies for 1.4kb inserts.

PCR master mix for screening 20 white colonies of each transformation:

			x 58
BM	10x PCR + Mg buffer (1x)	2.0 µl	116.0
BM	5u/µl Taq	0.2	11.6
	16µM Forward primer	0.15	8.7
	16µM Reverse primer	0.15	8.7
	50mM dNTP's	0.10	5.8
	H_2O	17.40	1009.2
		20.0	1160.0

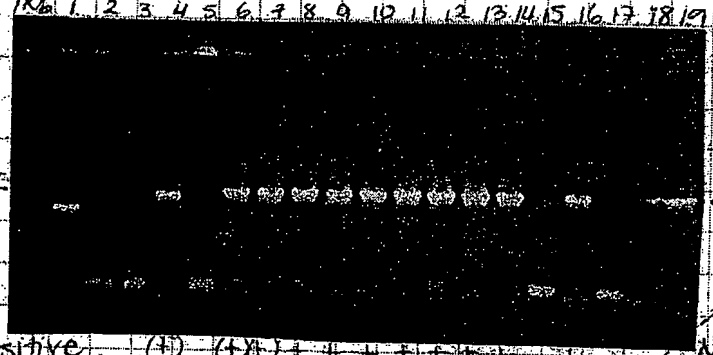
20µl / tube

Green = BEV 1+3 1-20 (incl. blue colony)
Blue = BEV 3+4 1-20 (incl. blue colony)

Program #26 9600 PE Plant lab.

15µl of each reaction loaded on 1% TAE gel
blue colony rxns (negative controls loaded onto another gel)

BEV 1+3 reactions 1-19

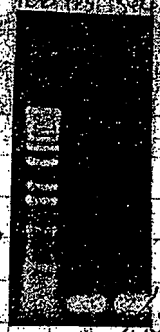


positive colonies: + + + + + + + + + + + + + + + + + + +

BEV 3+4 reactions 1-19

positive colonies: + + + + + + + + + + + + + + + + + + +

-ve controls - Clone coloured



Next: select colonies for setting up miniprep cultures. O/N cultures set up (circled positive colonies above)

Continued on Page #: 64 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
A. Bernard	P Campbell	M. Graham
NB 16/3/98 Dated	Campbell 16/3/98 Signed Dated	24/3/98 Dated

Page 64

Title of Experiment: EXPT. 11 cont.

MINIPREPS : PUTATIVE CLONES PCR2.1+BEV1-3 1.4Kb (PCR
PCR2.1 + BEV3+4 1.4Kb (PCR

Date 17/3/98

Continued From Page # 63 Book # 32

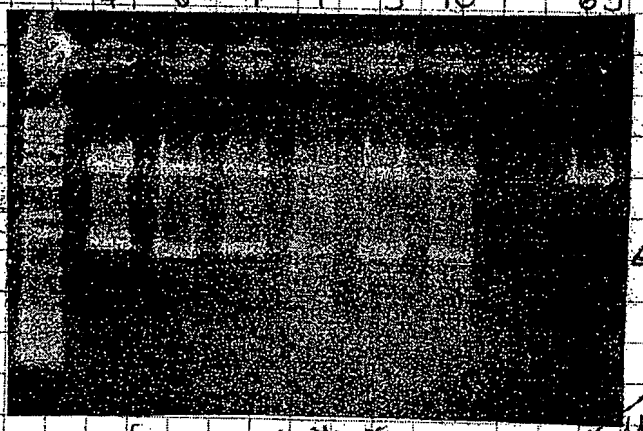
Qiagen spin columns
1.5ml culture, eluted in 100µl H_2O
5µl DNA digested with EcoRI

DNA	5.0µl
10X (H) buffer	2.0
EcoRI	1.0
H_2O	12.0
	20.0

done #
BEV1-3 4, 6, 7
BEV3+4 1, 5, 10
also included done #65 (see p. 57)
repeated digest to check restriction

15µl digest run on 1% TAE gel

PCR2.1+BEV1-3 4 6 7 | PCR2.1+BEV3+4 1 5 10 | PCR2.1+BEV3+4 6 5



kb
2.3
1.6

← 1.4Kb insert

KMB 17/3/98

- all of the clones have a 1.4Kb insert (except result on p. 57 with earlier digest of clone #65 see)
- clone #6 of PCR2.1+BEV1-3 → insert looks slightly smaller than with clones #4 #7.

Next: sequence clones with universal forward and reverse primers.

Continued on Page # 67 Book # 32

Name of Person Conducting Experiment: M. Bernard
Signed: MBernard Dated: 17/3/98

First Witness of Experiment: P. Campbell
Signed: Campbell Dated: 17/03/98

Second Witness of Experiment: M. Cowles

EXPT. 14

CLONING: PREPARATION OF pCMV.cass

Page 65

Date 13/3/98

Continued From Page #: Book #:

Aim: preparation of pCMV.cass

↓

pEGFP-N1-MCS NotI/PinAI deletion

fill in ends with Pfu polymerase

↓

religate

2/2R

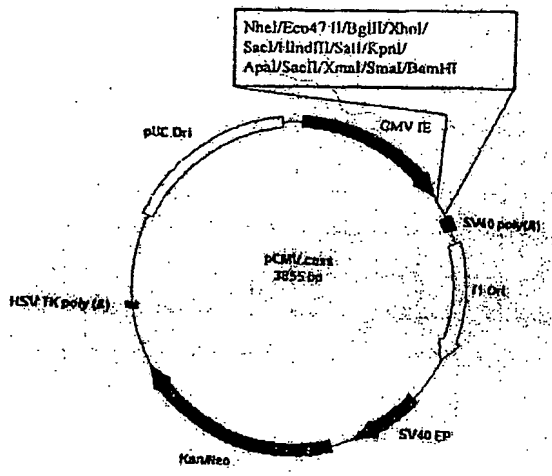
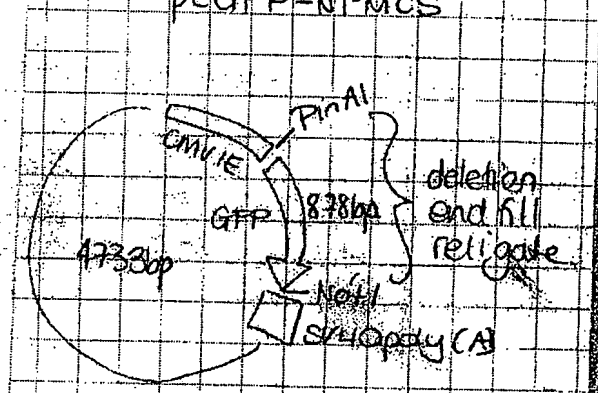


FIGURE 2.



NR 18/3/98

Method:

pEGFP-N1-MCS

DNA(midi)	15µl	inc. 37°C / 2 hrs
NotI	1·5 2·0	↓
10x buffer H	5·0 10·0	ppt'd DNA 2·5 vol 100% EtOH + 1/10 vol Na.
H_2O	28·5 / 44·5	acetate 1x 70% wash
	50·0 100·0	resuspended in 15µl H_2O (0·5µl for gel)

Next digest: 14·5µl DNA/NotI + 5µl 10x buffer B, 1·5µl 10µl PinAI +29µl H_2O = 30µl total

inc. 37°C 1·5hrs.

2µl aliquot for gel.

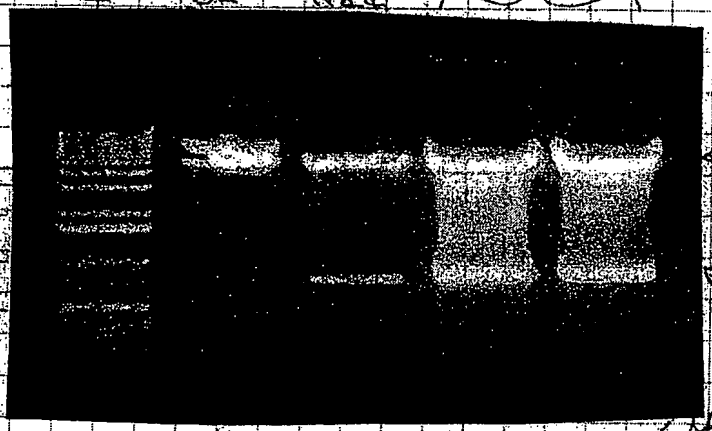
Continued on Page #: 66 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
S.M. Bernard	P. Campbell	M. Cudmore
SMBernard 18.3.98 Dated	Campbell 18/03/98 Signed / Dated	24.3.98 Dated

Pfu treatment of pEGFP-N1-MCS / Not I + Pin AI

48 µl digested DNA
1 µl 10mM dNTP's
6 µl 10x Pfu buffer (Stratagene)
1 µl Pfu polymerase " (cloned Pfu pol)
H2O 4 µl
60 µl total

72°C / 30' using 480 Perkin Elmer PCR machine
↓
total amount run on 1% TAE agarose gel



18/3/98

Gel purification of 3.8Kb bands:
approx wt. of gel fragments ~0.8g = 800µl
used QIAquick Gel purification kit
eluted in 20µl H2O & set up ligation

Ligation

7.5µl DNA
1.0µl ligase
1.5µl 10x lign buffer } BM inc. @ RT O/N
5.0µl H2O
15.0µl

Continued on Page #: 68 Book #: 32

Name of Person Conducting Experiment: M. Bernard
First Witness of Experiment: P. Campbell
Second Witness of Experiment:

M.Bernard 18/3/98 (Signed / Dated)
P. Campbell 18/03/98 (Signed / Dated)

EXPT. 15

SEQUENCING: PCR.BEV.2 clones #4 + #7
PCR.BEV.3 clones #1 + #5

Page 67

Date 18/3/98

Continued From Page #: 64 Book #: 32

Aim: to sequence 2 clones of each to check BEV inserts.

Method: new primers arrived (x Derek Skingle)

		x 4 for each primer
Big Dye	8.0 µl	32.0
16µM primer	0.2	0.8
DNA (miniprep)	4.0	–
H_2O	7.8	31.2
	20.0	64.0

seq. rvn			Primer	label
1	PCR.BEV.2	clone #4	F	QMG 40
2	"	"	R	41
3	"	clone #7	F	42
4	"	"	R	43
5	PCR.BEV.3	clone #1	F	44
6	"	"	R	45
7	"	clone #5	F	46
8	"	"	R	47

LM 18/3/98

Clean up: 2µl 3M Na acetate
50µl 100% etOH (RT)
↓
-20°C / 12 hrs.
↓
14k / 30' @ 4°C
↓
1 x 70% wash
↓
Speedivac to dry pellet

NB. not all volumes after sequencing run looked equal. preparing a master mix may not be very good!

Continued on Page #: Book #:

Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P. Campbell	M. Graham
18/3/98 Dated	19/3/98 Campbell Signed	19/05/98 Dated
		24/3/98 Dated

Page 68

Title of Experiment EXP1.14 cont.

TRANSFORMATIONS: pCMV.cass

Date 19/3/98

Continued From Page # 66 Book # 32

Aim: to transform ligation from p.66 into DH5α

Method:

cells	Amt. ligation
DH5α chemically competent	
① 50µl	5µl
② 50µl	10µl

Heat shock protocol.
Recovery in 450µl SOC

plated out 2x100 ~~+200~~µl onto LB+kan 50µg/ml. 19/3

20/3/98

Transformation # colonies
1 19 + 12
2 22 + 15

Next: set up miniprep cultures (LB+kan 50µg/ml

22/3/98

12 colonies selected + miniprep cultures set up
2ml LB+kan 50µg/ml
Inc. 37°C O/N.

Continued on Page # 69 Book # 32

Name of Person Conducting Experiment | First Witness of Experiment | Second Witness of Experiment

M. Bernard | P. Campbell

M.Bernard 22.3.98 Signed Dated | P. Campbell 22/03/98 Signed Dated

Expt. 14 cont.

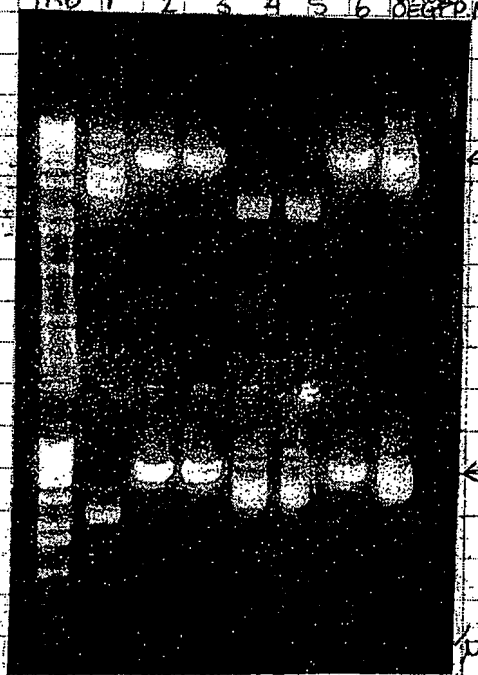
MINIPREPS : PUTATIVE pCMV.cass CLONES

Qiagen spin columns
eluted in 100µl H_2O
5µl DNA digested with NotI
↓
clones (if correct) should have lost the NotI site after end filling with Pfu & religating : correct clones should run as uncut vector, but smaller (i.e. faster)
Digests:

		x 12
DNA	5.0µl	
10x buffer H	2.0	24.0
10u/µl NotI	0.5	6.0
H_2O	12.5	150.0
	20.0	180.0

INC 37°C / 2 hrs

Total amount of digest run on 1% TAE agarose (+ETBr):



←4.2Kb linearised pEGFP-N1 MCS

←4.2Kb linearised pEGFP-N1 MCS

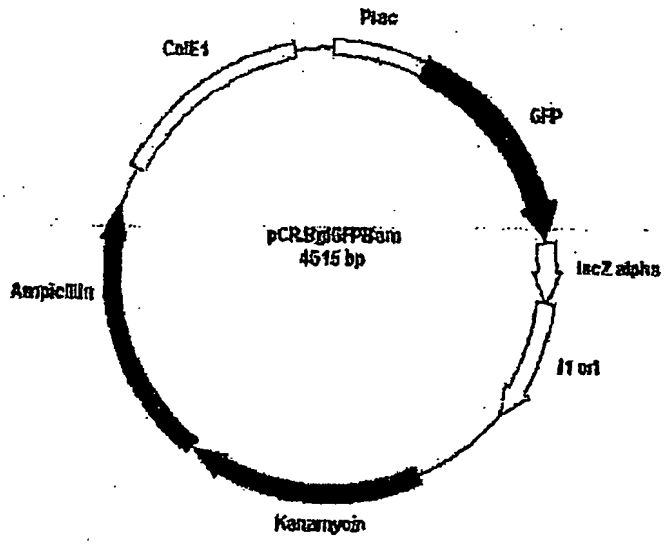
JMA 23.3.98

Comments: clones #4, 5 + 7 are running as uncut & faster than control → check further with other RE
- clone #1, 10 & 11 are running as uncut & only slightly faster than control → maybe check one of these clones.

Continued on Page #: 70 Book #: 32

Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
Bernard	P. Campbell	M. Graham
Bernard 23/3/98 Dated	Campbell 23/03/98 Signed / Dated	23/3/98 Signed / Dated

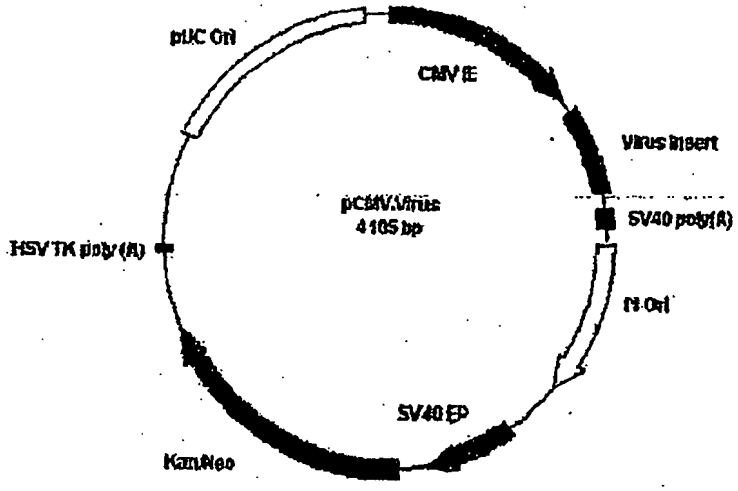
EXHIBIT 8



Author:
Date:
Notes:

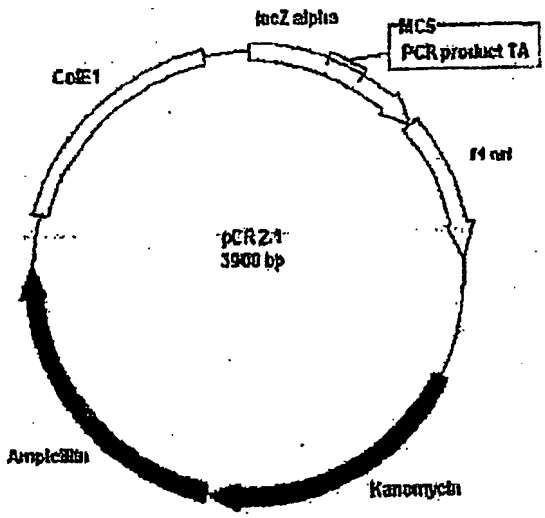
PBG40LBA-PLA

Created 21/01/1998

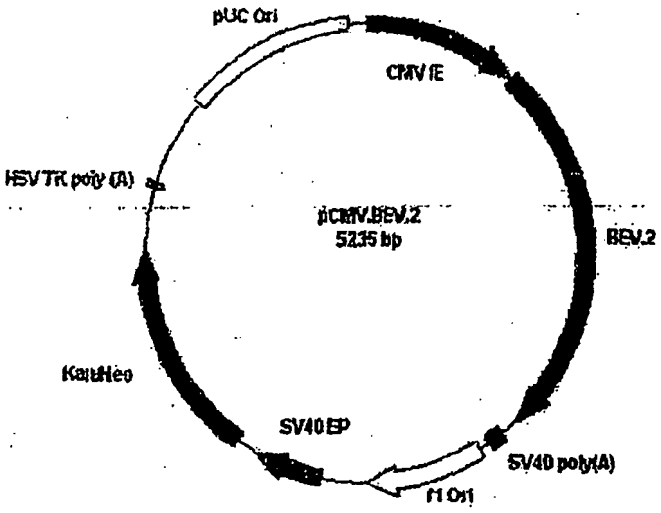


Author:
Date:
Notes:

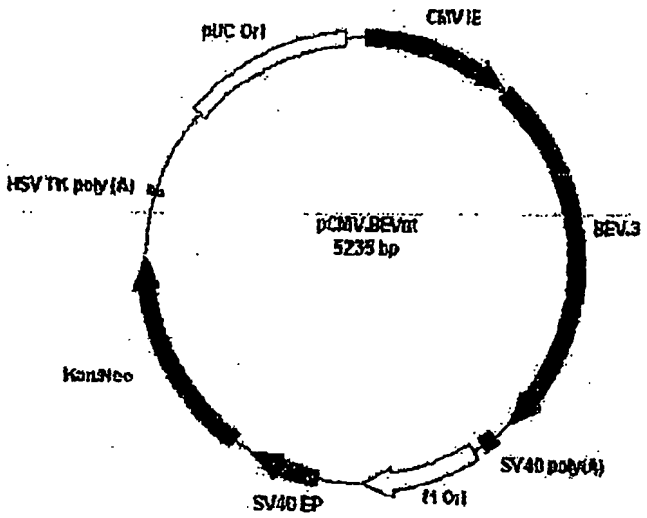
PCMV_VIR.PLA
Created 21/01/1998



Author: PLR2_1.PLA
Date:
Notes: Created 21/01/1998

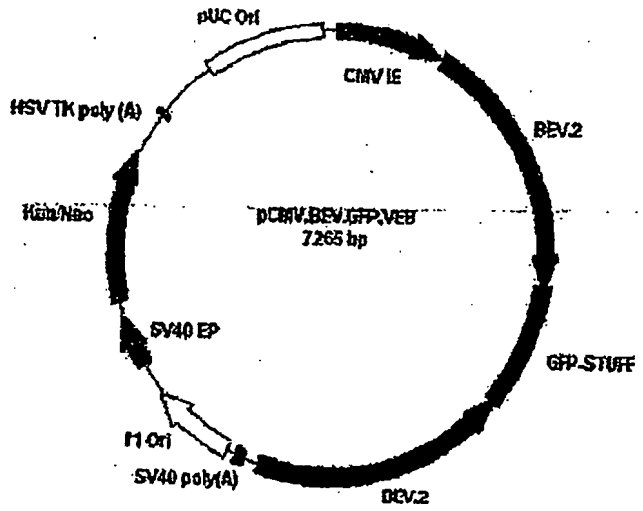


Author: PCMVBEV2.PLA
Date:
Notes: Created 22/01/1998



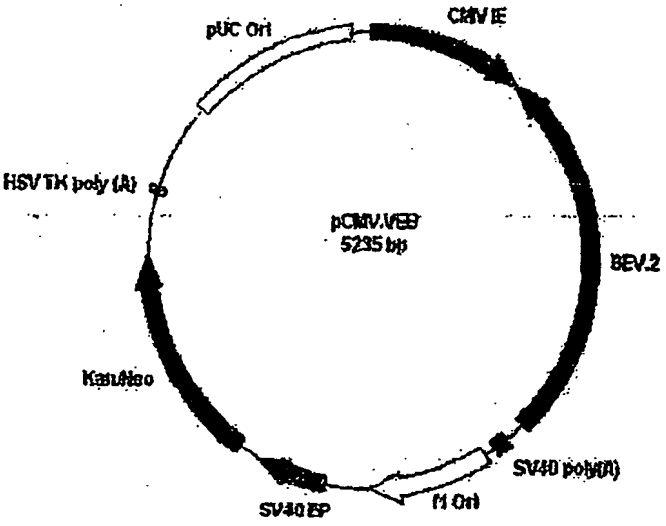
nt = Non-translatable

Author:
Date:
Notes: PCMVBEV3: PLA
created 22/01/1998



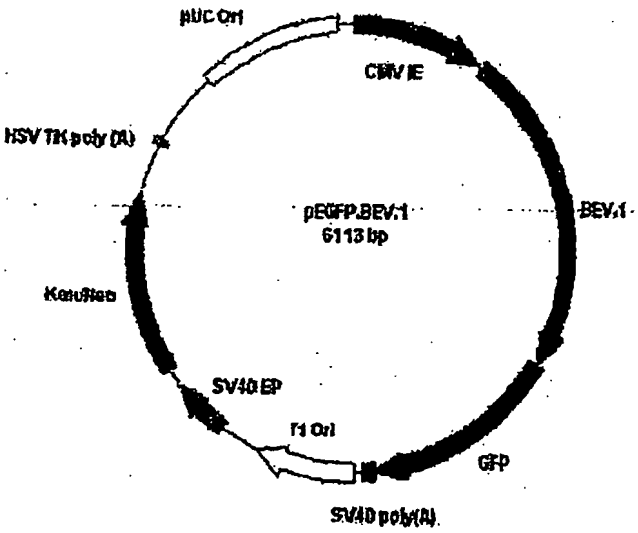
Author:
Date:
Notes:

PCMVBGV.PLA
Created 22/01/1998



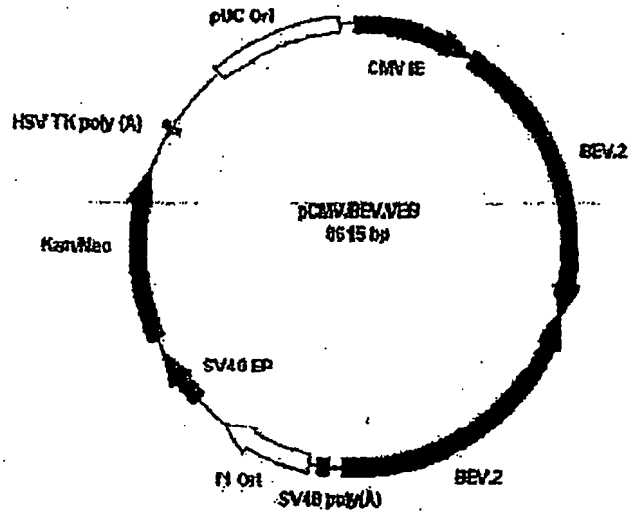
Author:
Date:
Notes: PCMVVEB2.PLA

Created 22/01/1998



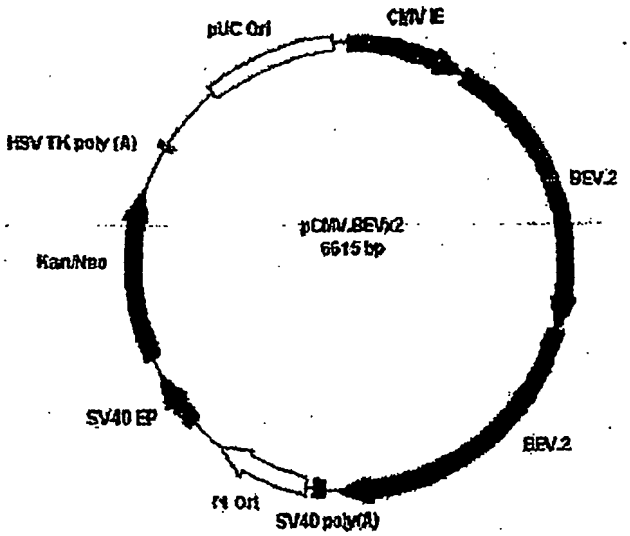
Author:
Date:
Notes:

PEGFPBEV.PLA
created 22/01/1998



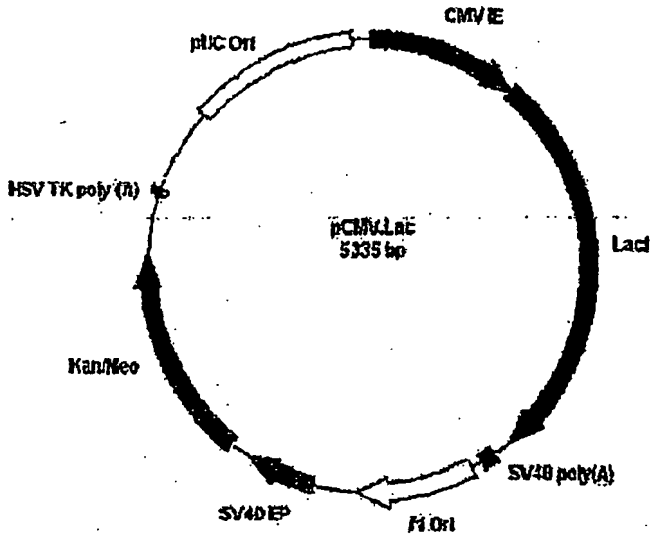
Author:
Date:
Notes:

PCMV BEV.VEB
Created 22/01/1998



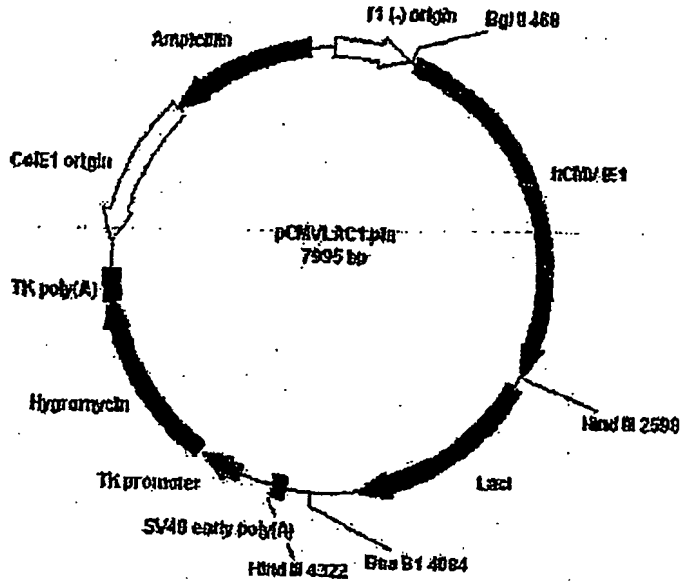
Author:
Date:
Notes: pCMVBEV2.X2

created 22/01/1998



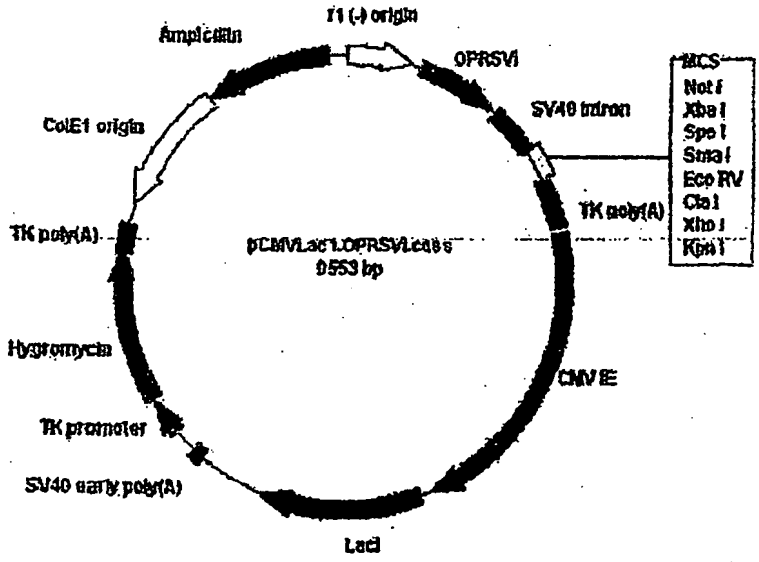
Author:
Date:
Notes:

PCMV_LAC.PLA
created 25/02/1998

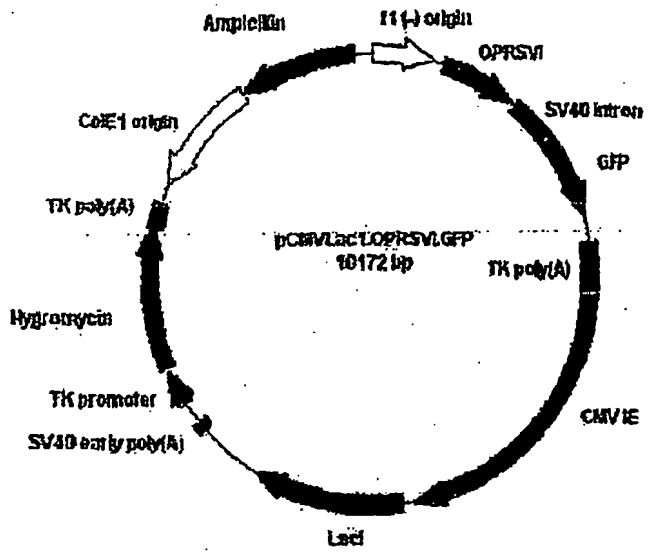


Author:
Date:
Notes:

PCMVLACI.PIA
created 25/02/1998



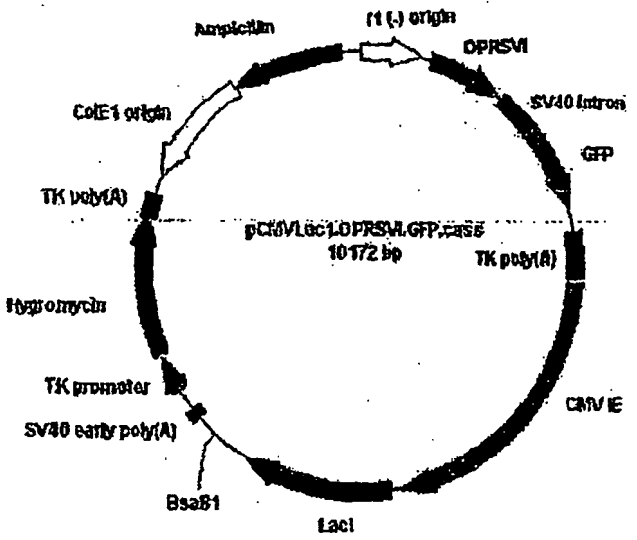
Author: PCMVORRS.CAS
Date:
Notes: Verified 26/02/1998



Author:
Date:
Notes:

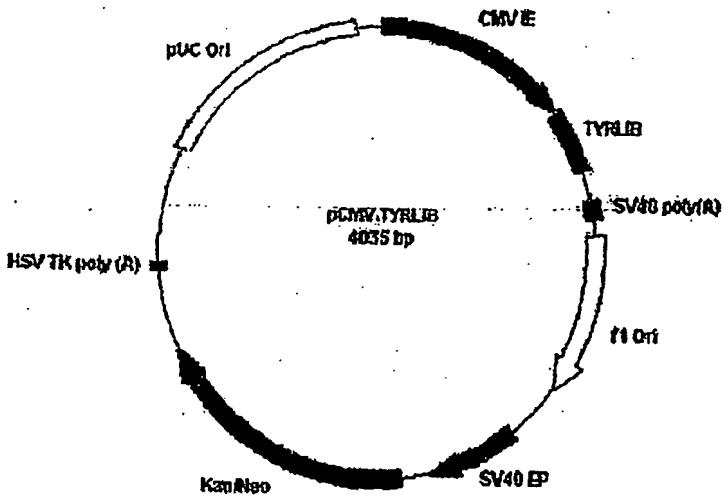
CMOPRGFP. PLA

created 26/02/1998



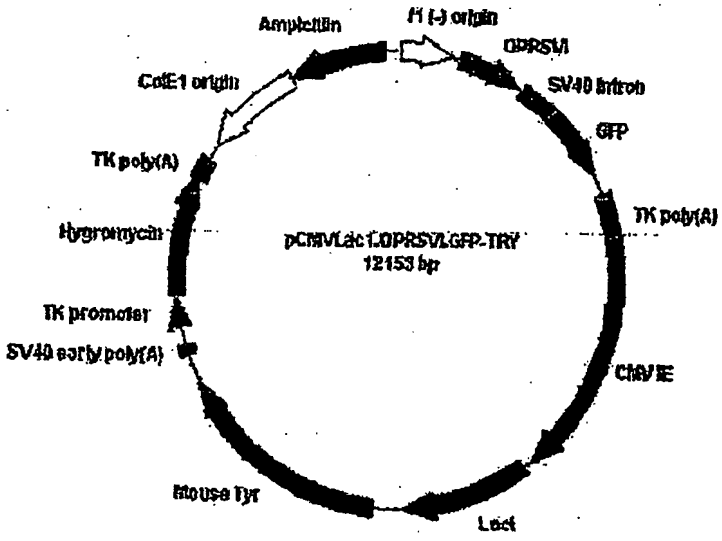
Author:
Date:
Notes: CMOPRGFP.CAS

created 27/02/1998

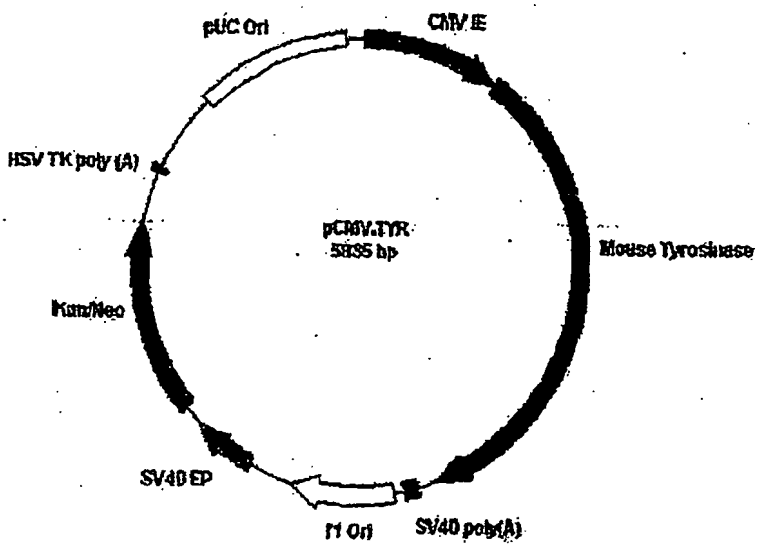


Author:
Date:
Notes:

CMVTRYLI.PLA
Created 27/02/1998

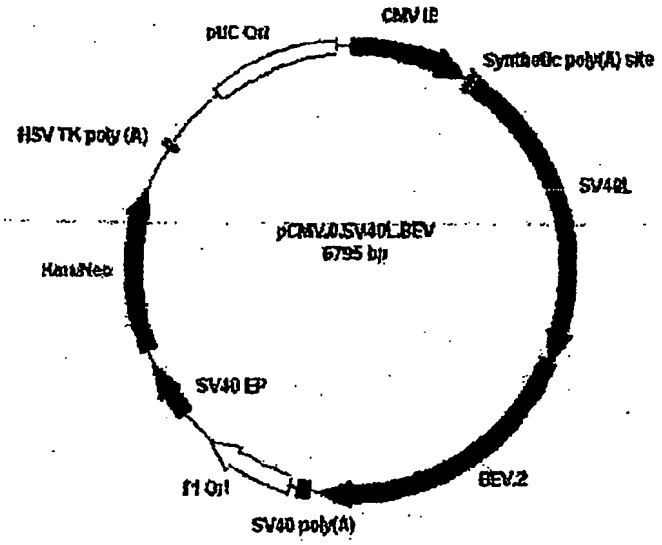


Author: CMOPRGFT.PLA
Date:
Notes: Created 27/01/1998

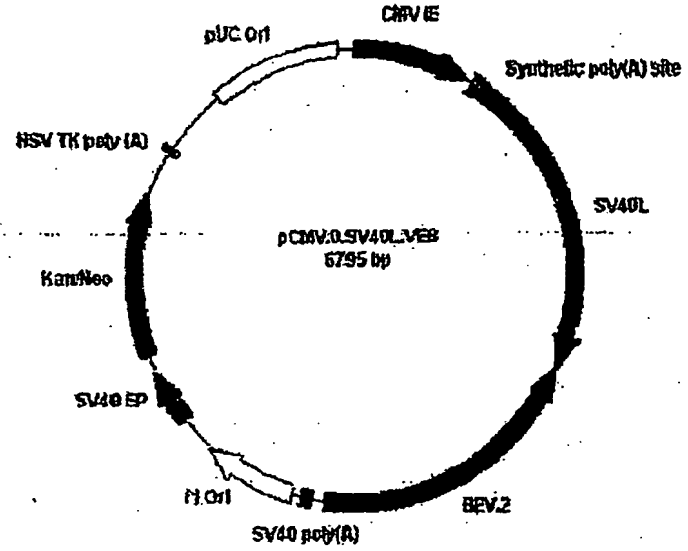


Author:
Date:
Notes:

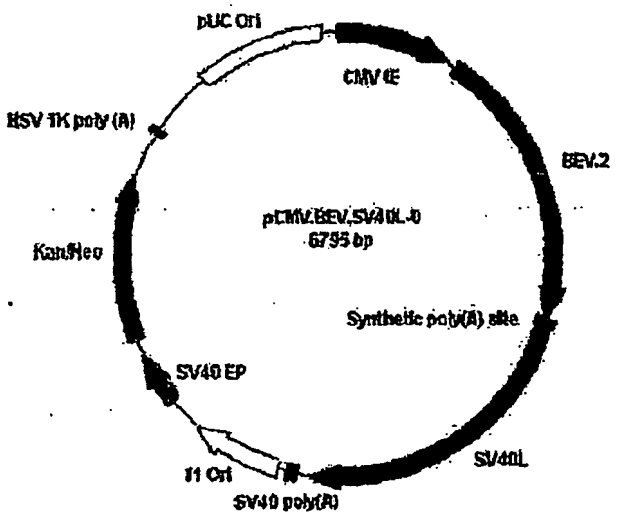
CMVTRY.PLA
Created 2/03/1998



Author:
Date:
Notes: File 05V40BE.pla
Created 5/03/1998



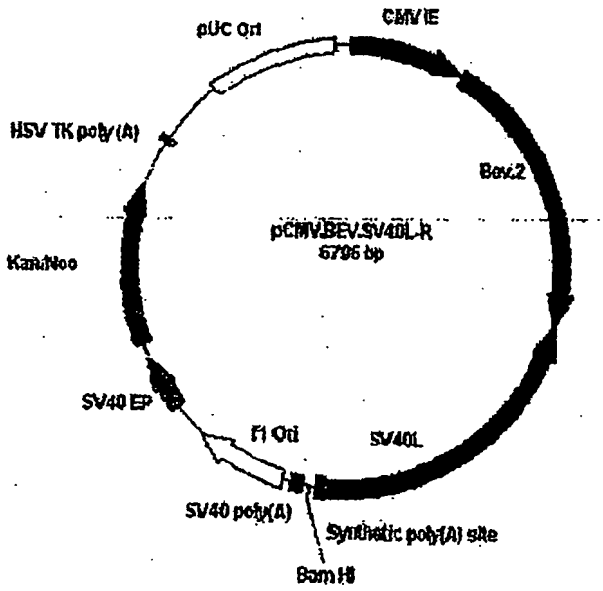
Author: OSV40VEB. P/a
Date:
Notes: Created 5/03/1998



Author:
Date:
Notes:

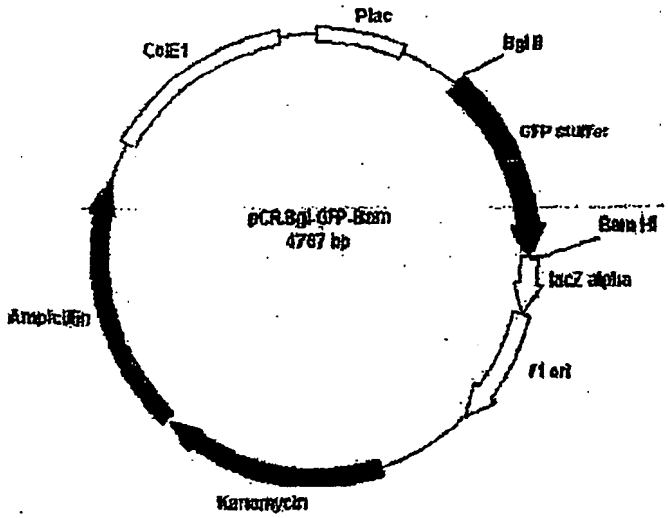
BE_S40_0.pla

Created 5/03/1998

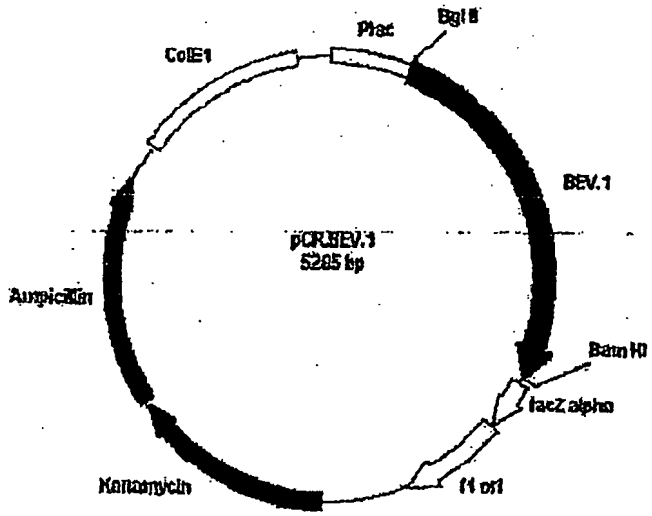


Author:
Date:
Notes:

CMBEV40R. PLA
Created 5/03/1998

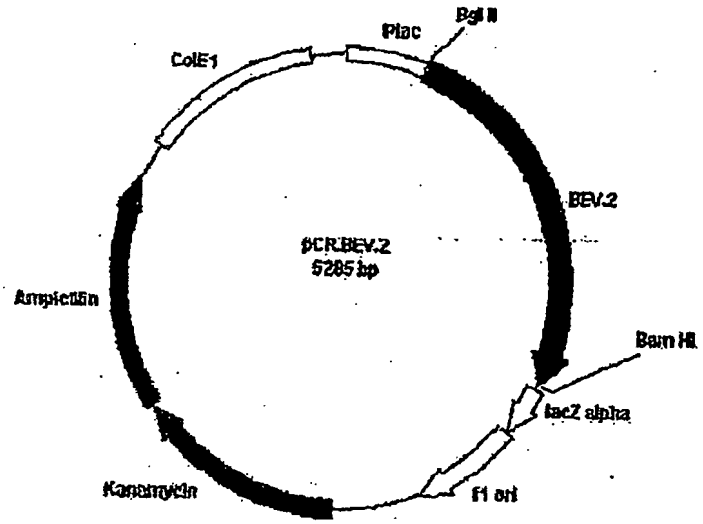


Author: PCBGFPBA.PLA
Date: created 5/03/1998
Notes:



Author:
Date:
Notes:

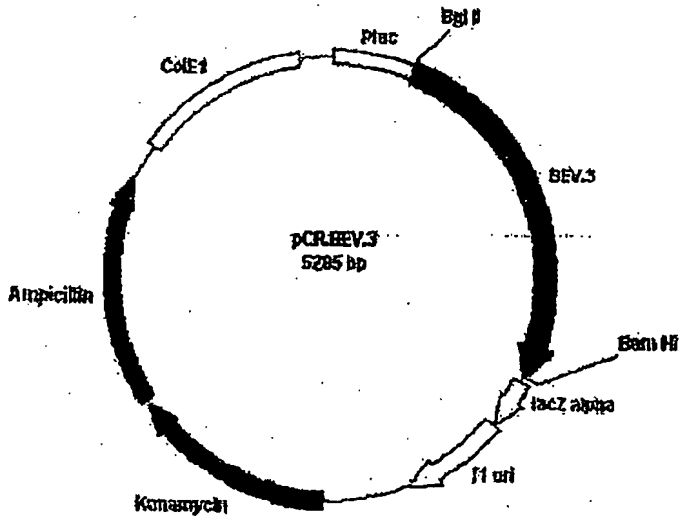
PCR_BEV1.PLA
created 5/03/1998



Author:
Date:
Notes:

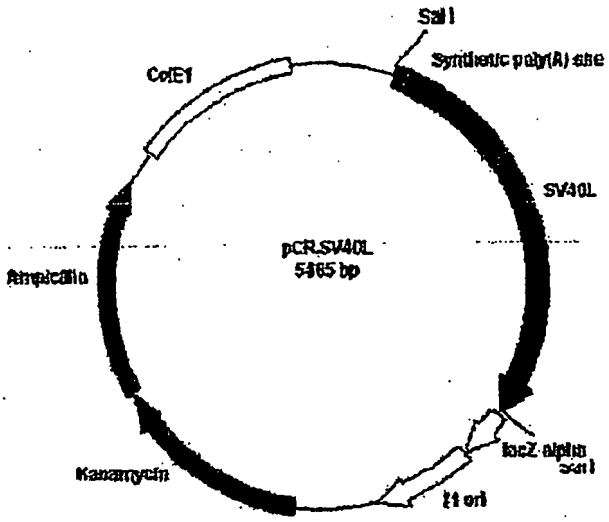
PCR_BEV2.PLA

created 5/03/1998



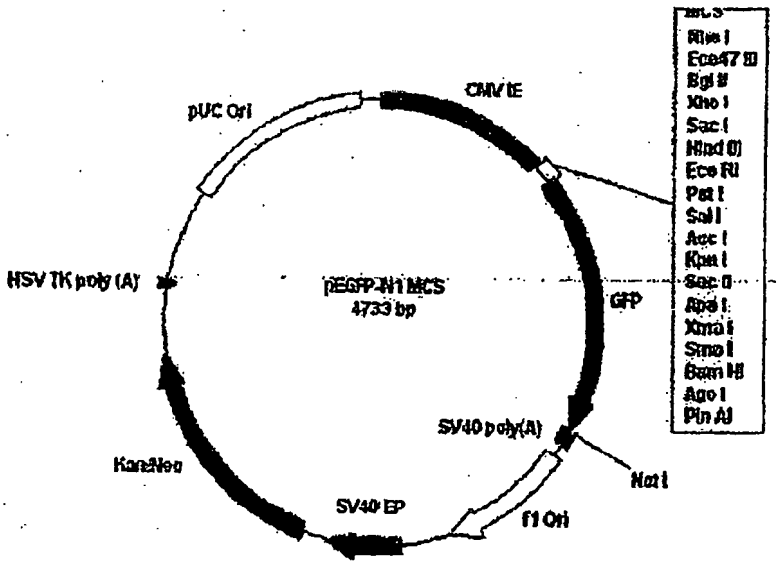
Author:
Date:
Notes:

PCR_BEV3.PLA
created 5/03/1998



Author:
Date:
Notes:

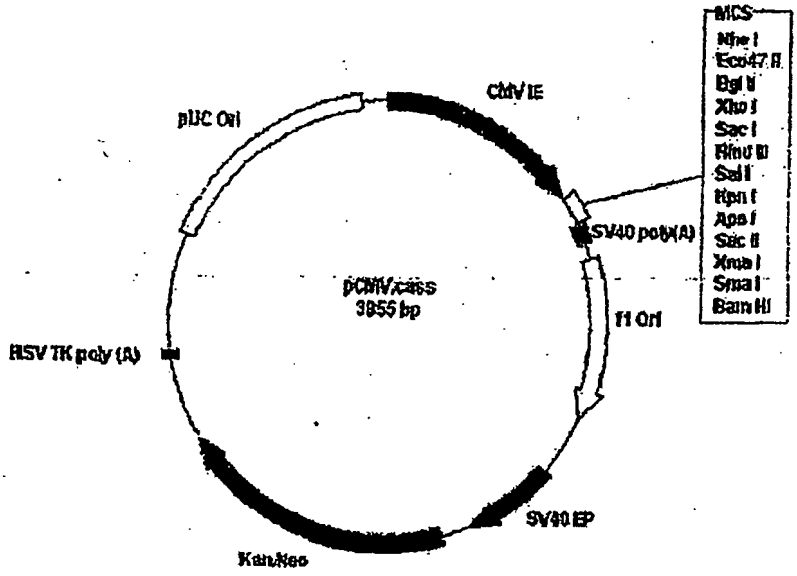
pCR SV40L. PLA
created 5/03/1998



Author: Robert Rice
Date: 22/1/98
Notes:

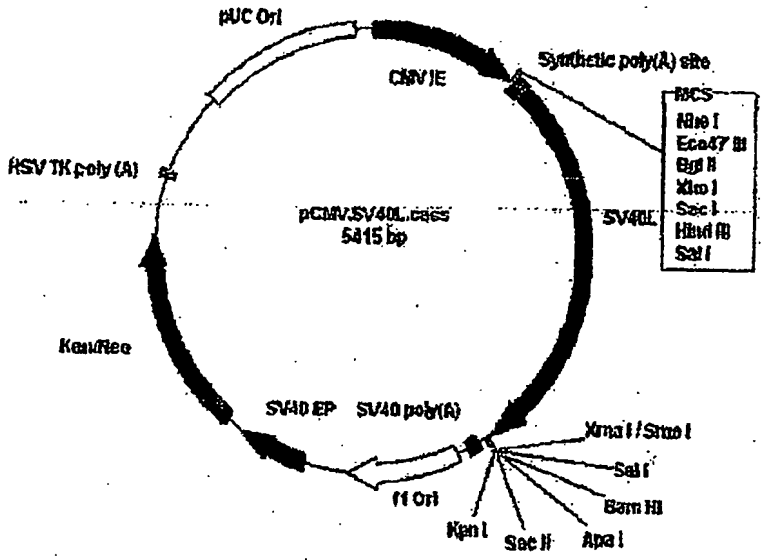
PEGFP-N1.PLA
Created 5/03/1998

Expression cassette: pEGFP-N1MCS: A commercially obtained vector (CLONTECH) from which most expression constructs are be derived..
to



Author:
Date:
Notes: PCMV.CAS

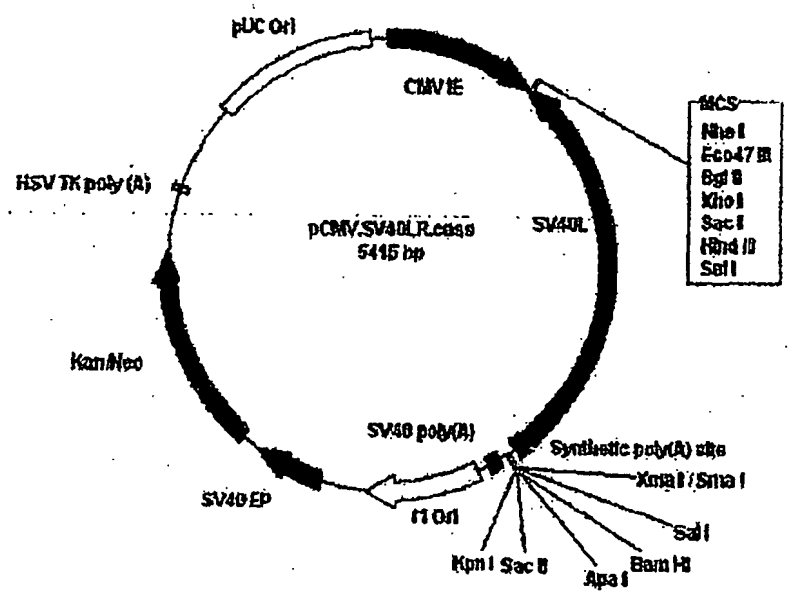
created 6/03/1998



Author:
Date:
Notes:

PCMV SV40. CAS

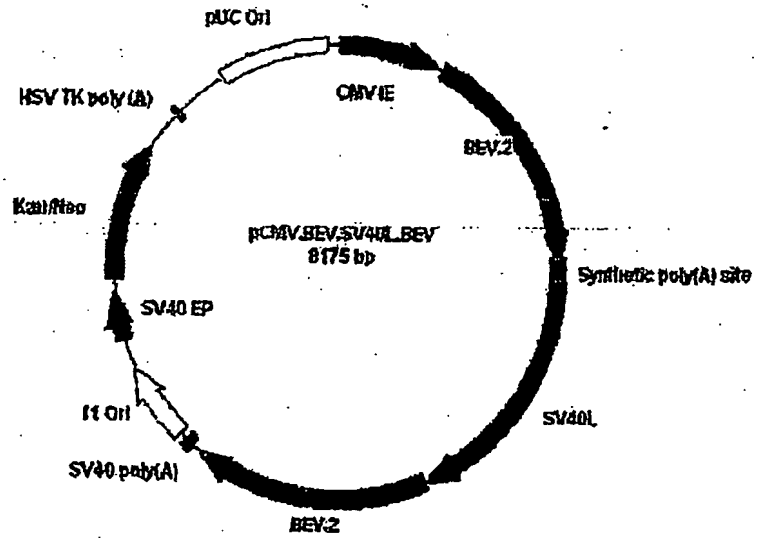
Created 6/03/1998



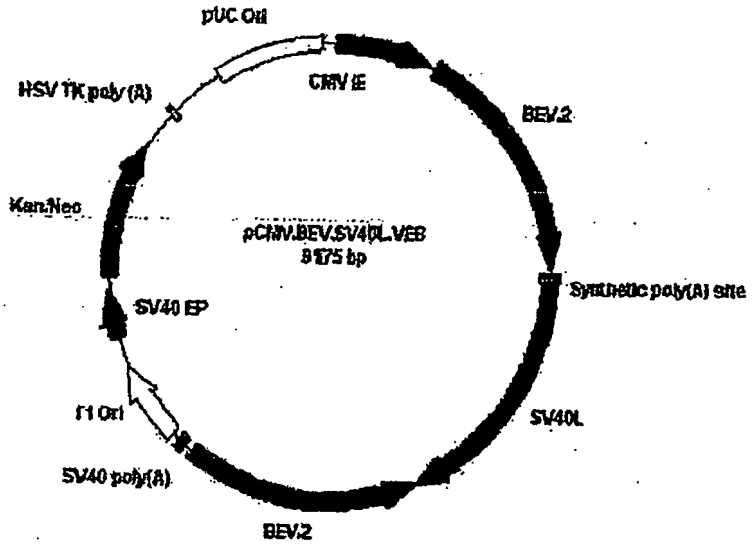
Author:
Date:
Notes:

PCMVSV4R.CAS

Created 6/03/1998

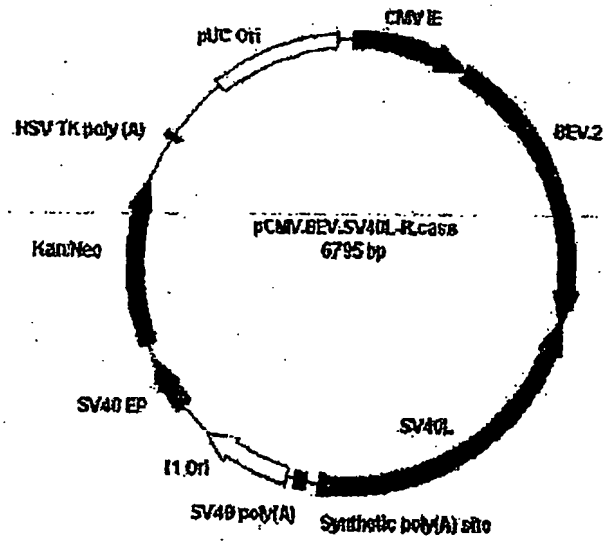


Author: BEVSVBEV.PIA
Date:
Notes: Created 6/03/1998



Author:
Date:
Notes:

BEVSVVEB.P1A
created 6/03/1998



Author: CMBE SV4R. PIA
Date:
Notes: Created 6/03/1998

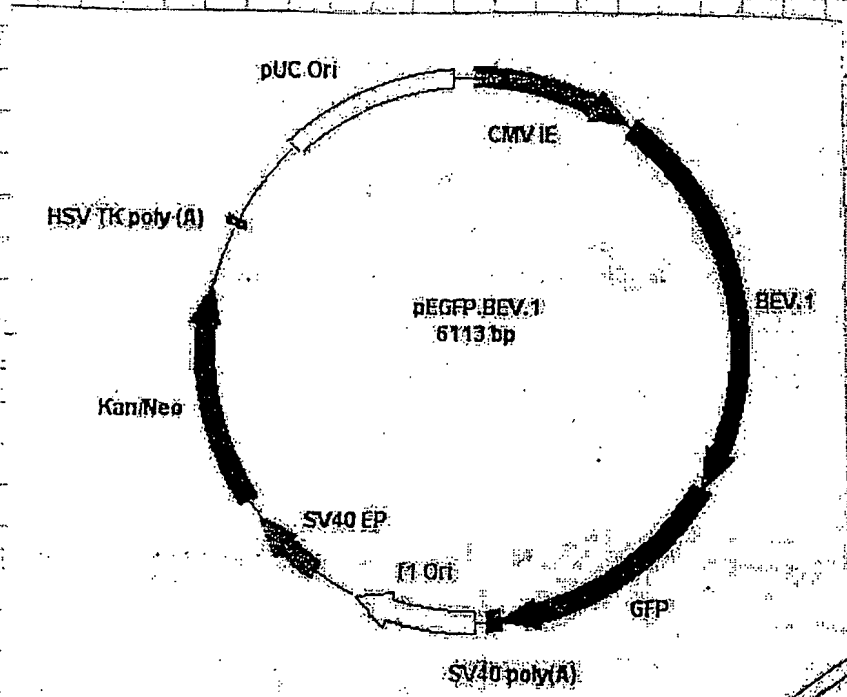
EXHIBIT 9

Experiment

Transfecting MDBK cells with pEGFP.BEV.1

Date 11/3/98

Continued From
Page #:
Book #:



Plasmids
BEV.1
GFP
Transfection protocol

11/3/98

From split MDBK cells on Monday 1/500 / passage into
For passages to weekly.

Set up transfections as follows:

Prepare lipofectamine: x ml DNA (1 mg)
100 x ml OPTI MEM
For each well: x ml (10 ul lipofectamine + 90 ul OPTI MEM)

18 wells	Transfect	Selection
wells		
1, 2	no DNA	none
3, 4	no DNA	Select drug of choice
5, 6	GFP control (pEGFP-C1/N1)	"
7-12	pEGFP.BEV.1	"
13-18	pEGFP.BEV.1	"

Continued on
Page #: 2
Book #: 41

Name of Person Conducting Experiment

Signed: 11/3/98
Dated

First Witness of Experiment

Signed: 11/3/98
Dated

Second Witness of Experiment

Signed: 11/3/98
Dated

Page 2

Title of Experiment: Expt 1

Date 1/1/-

Continued From: Page #: 1 Book #: 41

Mixes prepared

1-4 50ml Lipofectamine + 4ml OPTI-MEM
950ml OPTI-MEM

5-6 30ml Lipofectamine + 2.4ml OPTI-MEM
570ml MEM OPT
4ml FCS + W1 MCS (2.2ml)

7-12 and 13-16 70ml Lipofectamine + 5.6 ml
1,330ml OPTI-MEM
Tube FCS + W1 MCS
RED CAPS

Each mixes ~1.5hrs before adding 2ml aliquot of media

Cells washed 2 x 1ml OPTI-MEM (1-12)
2 x 0.5ml (13-16)

Added 1ml DNA mix from above

Leave 2hrs then add media

50ml MEM
25ml FCS
5ml non-essential aa
5ml antibiotics
15ml Hi-Pro-Lipofectamine

Continued on: Page #: Book #:

Name of Person Conducting Experiment: M. Gresham
Signed: M. Gresham Dated: 11/3/98

First Witness of Experiment: KC REED
Signed: KC Reed Dated: 11/3/98

Second Witness of Experiment: FIONA MCCARTHY
Signed: Fiona McCarthy Dated: 11/3/98

Name of Experiment: Expt 1

Date: 17/3/98

Continued From: Page #: Book #:

16/3/98 Changed Removed OPTI media, replaced with fresh MEM.
13/3/98 Changed media for selection.
(Geneticin 330ml/40ml MEM)

1-2 normal MEM
3-18 MEM + geneticin

GFP staining

5 + 6 heaps of GFP pos cells
(~10/field) looks fine

7 - 18 GFP pos cells apparent - very few (< 10x less) than controls

Human factor not as active as native factor or something else (Wnt shh???)

Will change media every 2 days + monitor selected

16/3/98
Cultured fibrin
Cells washed 2x with PBS
Treated ± 2ml trypsin - 10min
Cultured on 2 fibrins 300,000 cells ⇒ 40ml MEM

For experiments cultured 6x6 well plates
0.36ml cells + 180ml media

17/3/98 Changed media for expt 1
Selection looks fairly good.

Continued on: Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M Gardiner	Tim O'Hare	P Campbell
Signed	Signed	Signed
17/3/98 Dated	20/3/98 Dated	30/03/98 Dated

Page 4

Title of Experiment

Expt 2: Kill curve for quenching of 1000 BK cells

Expt 3: Direct selection for BEV immunity

Date 12/3/04

Continued From Page #: Book #:

2 transformations

Expt 2, 3 plates pCAGGS-U1 MCS, Ampicillin

Expt 2 " pCAGGS BEV GPC

Prepare DNA for 20 TIF

DNA	Expt 2 Xbal pCAGGS-MCS	Expt 3 Xbal pCAGGS BEV	
Ligation mix	200 ul	200 ul	Streaked out dead cells on LB/Amp plates
OPTI MEM	1.7 ml	1.95 ml	
	45 in RT		
LB	Xbal OPTI MEM	Xbal OPTI MEM	Pooled empty transfected GPC

Wash cells 2x Xbal OPTI MEM

Add 1 ml of above mixes to plates

Expt 1 Kill curve	ug/ml Ceocin	Ml Ceocin/Xbal media	Start 30 ug/ml
1,2	0	0	
3,4	200	3	
5,6	300	4.8	
7,8	400	6.4	
9,10	500	8.0	
11,12	600	9.6	
13,14	700	11.2	
15,16	800	12.8	
17,18	1,000	16.0	

Continued on Page #: Book #:

Name of Person Conducting Experiment	First Witness of Experiment	Second Witness of Experiment
M. Carlson	P. Campbell	
Signed	Signed	Signed
12/3/04	20 12/3/04	20 12/3/04
Dated	Dated	Dated

Page 5

Date 20/2/98

Continued From Page #: Book #:

Change media for Expt 1

2
3

Expt 1 1,2 change media to granulocyte
3-K " " + HGF/IL granulocyte
Tome media + ~~250 ul~~ 500 ul granulocyte

Expt 2 Kill curve, as on previous page

Expt 3 Change media, will inject cells Monday with PEU

Continuation Page #: Book #:

Name of Person Conducting Experiment | First Witness of Experiment | Second Witness of Experiment

M. Gavahan | P. Campbell | [illegible]

Signed | 20/3/98 Dated | Campbell 20/03/98 Signed Dated | Signed | 20/3/98 Dated

EXHIBIT C

Docket No.: 023004.0103X1US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reexamination Application of:
Michael W. Graham et al.

Application No.: 90/007247 — Confirmation No.: 6310

Filed: October 4, 2004 — Art Unit: 1639

For: GENETIC CONSTRUCTS FOR DELAYING OR REPRESSING THE EXPRESSION OF A TARGET GENE — Examiner: B. M. Celsa

DECLARATION UNDER 37 C.F.R. § 1.131

Customer Window, MS Amendment
U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

Dear Sir:

I, Kenneth Reed, Ph.D., declare as follows:

1. I am a resident and citizen of Australia. From early 1997 through to the filing of the priority document ("relevant time period") for the patent under reexamination, I was the Director of the Queensland Agricultural Biotechnology Centre (QABC), an operational centre of the Queensland State Government's Department of Primary Industries (DPI). Further, I was an observer on the board of directors for Ag-Gene Pty Limited, which subsequently became Benitec Limited, during this period of time.

2. The laboratory facilities of Ag-Gene were located at the QABC from early 1997 until after the filing of the priority document for the patent under reexamination. During this period of time, all full-time research employees hired by Ag-Gene (such as Robert Rice) and DPI employees who conducted research for Ag-Gene and whose salaries were paid in part by Ag-

Gene (such as Michael Graham and Margaret Bernard) worked in the laboratory facilities at QABC.

3. As the former Director of QABC I am knowledgeable about the operations of QABC and when the facilities were opened or closed during the relevant time period.

4. As an observer on the Board of Directors of Ag-Gene, I am knowledgeable about the employees hired by Ag-Gene, when they were hired, for what purpose they were hired, and under whose direction they worked during the relevant time period.

5. I have reviewed the above-identified reexamination, including the present claims. As I understand it, the presently claimed subject matter is generally directed to genetic constructs that are capable of delaying, repressing or otherwise reducing the expression of a target gene in an animal cell, as well as methods for using these constructs and animal cells comprising these constructs. I understand that the presently claimed constructs comprise at least one structural gene sequence placed operably in a sense orientation under the control of a promoter and at least one structural gene sequence placed operably in an antisense orientation under the control of a promoter, where the structural gene sequences comprise a nucleotide sequence which is substantially identical to at least a region of a target gene, and where

a. the multiple structural gene sequences are placed operably under the control of a single promoter sequence, where optionally the structural gene sequences in sense and antisense orientations are spaced from each other by a nucleic acid stuffer fragment; or

b. the structural gene sequences in sense and antisense orientations are each placed operably under the control of individual promoter sequences.

6. Exhibit 1 is my electronic diary entry of October 23, 1997 ("23/10/97"). This entry is related to a teleconference between myself, Geoff Lambert (at that time the Managing Director of Ag-Gene) and John Hunt (at that time a Non-Executive Director of Ag-Gene). The diary entry states "Robert Rice re co-suppression (MWG)." The purpose of this entry was to remind

myself to discuss prospects of hiring Robert Rice, an inventor of the patent under current reexamination, to study co-suppression in animal cells under the supervision of Michael Graham, *i.e.*, "(MWG)."

7. From what I recall, we wanted to hire someone with extensive experience in a range of molecular biological techniques and eukaryotic plasmid design and construction to make a series of genetic constructs that correspond to the invention that is referred to in paragraph 5 above and that were later included in the patent application now under re-examination. I reviewed Dr. Rice's C.V. in November of 1997 to determine his skill set. A copy of Dr. Rice's November 1997 C.V. is attached as Exhibit 2. Dr. Rice had the experience in eukaryotic plasmid design and construction that we were looking for. Further, his thesis topic was eukaryotic evolution and a study of eukaryotic divergence using ribosomal RNA sequence data and secondary structure remodeling. As such, Dr. Rice had experience with use of computers for systematic / bioinformatics analysis of DNA / RNA sequences. Ag-Gene decided to hire Dr. Rice sometime in November 1997 and extended an offer, which he accepted.

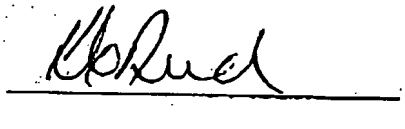
8. Dr. Rice arrived in Australia to start work at Ag-Gene on December 21, 1997. As I mentioned, the laboratory facilities of Ag-Gene were located at the QABC, an operational centre of the Queensland State Government's Department of Primary Industries. The Queensland State Government provided paid leave for Christmas day (December 25), Boxing Day (December 26) and New Year's Day (January 1). Further, the Queensland State Government mandated that all State Government employees do not work on the days between December 26 and January 1 and that such days must be taken as part of employees' annual leave entitlement. As such, the QABC laboratories and offices were closed from December 25 – January 1, 1997, inclusive. No entry to the QABC laboratories by any individual was permitted throughout that period for Government-mandated safety reasons. Further, it was customary in 1997/1998 for employees to take as leave Christmas Eve, December 24 and other days into early January.

9. It is my understanding that upon arrival Dr. Rice, under the supervision of Dr Graham, started researching the phenomenon of co-suppression in plants and designing a variety of DNA constructs to be used in animal models. It is my understanding that since the actual laboratory

facilities were not open over Christmas and into early January, 1998, Dr. Rice and Dr. Graham spent the period between December 22, 1997 and mid-January 1998 meeting to discuss co-suppression and DNA construct designs.

10. It is also my understanding the work of Dr. Rice and Dr. Graham narrowed down the exemplary constructs and Dr. Rice designed the approximately 35 plasmid constructs attached as Exhibit 3 no later than the dates set forth in Exhibit 3.

11. I declare that all statements made of my own knowledge are true and all statements made on information and belief I believed to be true. I make this declaration with the understanding that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the patent.



April 23, 2007

Kenneth Reed, Ph.D. Date

EXHIBIT 1

16/10/97 9:00 AM Aquaculture Industry Development Meeting: Lani West to attend. Aquaculture Industry Development (South) Subprogram meeting with staff. Joondaburri Training Centre, BIARC. Mike Potter to confirm and send further details at a later date.
16/10/97 2:00 PM :
16/10/97 2:00 PM PLY to meet with Peter Neville:
16/10/97 5:10 PM SYD-BNE: AN136:
17/10/97 Biodiversity Convention Working Group on Biosafety: Montreal
17/10/97 PLY Acting Director:
17/10/97 KCR in London with Geoff Lambert:
17/10/97 11:00 AM PLY, MWG & KGD to discuss QABC IP:
17/10/97 3:30 PM Seminar - Mr Tim Smith: "Boron Deficiency of avocado" Room 323, Hartley Teakle Bldg
20/10/97 Kathleen Heffernan to start work at QABC: Trainee Executive Assistant
20/10/97 8:30 AM Meet with Michelle for mail:
20/10/97 10:00 AM Meet with Peer Schenk:
20/10/97 12:30 PM Lunch with Richard Lewis: General catch up
20/10/97 3:00 PM Meet with KCR staff:
21/10/97 1:00 PM QABC Senior Management:
21/10/97 4:30 PM :
21/10/97 5:30 PM QABC BBQ (Walmsley/Kirk): "Oh Deer - Contraceptives for Bambi?"
22/10/97 10:30 AM Alan Chang to meet with KCR : Re: scientific positions
22/10/97 2:00 PM QABC Scientific Meeting: KCR to talk on London & agrobacterium alternatives
22/10/97 3:30 PM Kim Cedrick: work performance & progression
23/10/97 Michelle on Rec Leave:
23/10/97 Vetaform to visit QABC: Colin & Tony
23/10/97 9:00 AM Patent Attorney: Co-suppression, benign selection, transgenic sterility in fish. Aust Provisional? USA? UK? patents & inventory all relevant patents, re business strategy. Robert Rice re co-suppression (AMG), Christina Ruddick (Rod Cake)
23/10/97 12:00 PM Teleconference (Ag-Gene Board Meeting): Geoff Lambert, John Hunt. Hire Patent Attorney to write
23/10/97 1:00 PM Keith Williams: "Proteome: The meat in the sanawich between genomics and combinatorial chemistry"; Room 228, Molecular Biosciences Building, UQ
23/10/97 1:00 PM Lunch: Colin Davis, Tony Gestier, Paul Simpson
23/10/97 2:00 PM :
23/10/97 2:00 PM Dr Doug Wright to visit QABC: Chairman, MCRC Advisory Group
23/10/97 5:00 PM Moore-Gwett phone:
24/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
24/10/97 8:00 AM Meet with Peter Young and Pam Swepson:
24/10/97 9:00 AM QABC Lab meeting:
24/10/97 11:00 AM Admin meeting: PY, KG, ML, NG, KCR
24/10/97 12:30 PM Brian King to meet with KCR:
24/10/97 3:00 PM Cheryl McCaffery at QABC: Uniquest (ex-Florigene; ref Janet Caffin); grains biotech workshop at Bribie? advice re John Hughes to handle phantom patents. need for knowledge of all relevant IP. Stressed need for IP manager in Ag-Gene; suggested contracting patent expertise if protection, enforcement and mangement of IP is not core business.
24/10/97 3:30 PM Seminar: "Some applications of molecular markers to sorghum breeding programs" David Jordan, Room 323, Hartley Teakle Bldg
24/10/97 4:00 PM Mick Graham wants KCR car:
25/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
25/10/97 8:00 AM Golf with Dwayne Kirk: St Lucia
25/10/97 4:00 PM Amanda & Dwayne's Wedding:
26/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
26/10/97 9:02 AM Golf at Nudgee: Mike Symons, John Williamson, Victor
27/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
27/10/97 2:00 PM Brian King:
27/10/97 3:00 PM KCR to meet with Warren Hoey:
28/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
28/10/97 Workshop at Bribie: "Strategic directions for grains, oilseeds, sugar and fibre crops"BIARC Conference Centre: send info to Bryan Whan
29/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
29/10/97 5:30 PM Brisbane Developmental Biology Seminar: CMCB Seminar room third floor, Ritchie Laboratories UQ St Lucia CampusGraham Kay -QIMR. TOPIC- Screening Blastocysts for Imprinted Genes.Beer and Pizza will be provided courtesy of life technology.
30/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
30/10/97 2:00 PM :
31/10/97 Ralf & Colleen in China: can be reached via email (ocgil@public.wh.hb.cn) and fax (0015-86-27-681 6451) of Professor Xu Zeyong at the Oil Crops Research Institute in Wuhan
31/10/97 8:00 AM Ian Jones: phone
31/10/97 9:00 AM QABC Lab meeting:
31/10/97 10:00 AM KCR Lab meeting:
31/10/97 11:00 AM Vivien McAnna to come to QABC: re:the possibility of Ithace Tafe being responsible for our computer maintenance. Vivien has expressed some interest in this and is sure we can come to some arrangements
31/10/97 12:00 PM CMCB seminar : Maize transposons and transgenic tomato: a powerful combination for cloning genes and regulatory sequences from plants.Centre for molecular and Cellular Biology, Seminar Room Level 3 Ritchie Research Building.
31/10/97 12:00 PM Marsupial CRC group meeting in KCR's office:
31/10/97 3:30 PM Seminar: "1.6 million sorghum crops" or "How does your sorghum grow" Prof. Richar Vanderlip, Kansas State Uni, Room 323, Hartley Teakle Bldg

EXHIBIT 2

CURRICULUM VITAE

Robert Rice

PERSONAL DETAILS

Address : Apicultural Service Manager (South Island)
Ministry of Agriculture
P.O. Box 24,
Lincoln,
New Zealand.
Telephone 64-3-3253920
Fax 64-3-3253918
E-mail ricer@lincoln.mqm.govt.nz

Home Address : House 62,
Lincoln University,
Lincoln, New Zealand.
Telephone 64-3-3253317

Date of Birth : 28th of October, 1958

Marital Status : Married

Citizenship : Australian

EDUCATION

QUALIFICATIONS AND TRAINING

Tertiary

1996 Doctorate of Philosophy
Thesis Title is "The Molecular Taxonomy of Two Microsporidia".
Australian National University, Canberra ACT
Submission Date 31/5/1996

1992 Bachelor of Science (Honours Degree)
Thesis Title : "The isolation of a putative alkaline protease gene from *Aspergillus nidulans*".
Awarded 1st Class Honours
University of New England, Armidale, New South Wales.

1991 Bachelor of Science (Majored in Genetics)
University of New England, Armidale, New South Wales.

1978 Associate Diploma in Rural Techniques (Apiculture)
Queensland Agricultural College, Lawes, Queensland.

WORK EXPERIENCE

1995-	Apiculture Services Manager, Ministry of Agriculture, Lincoln, New Zealand.
1993-94	Demonstrator (Casual), Biological Sciences Botany and Zoology Department Australian National University, Canberra ACT
1985-1988	Production Manager for Apiary Operations Rice's Aussie Bee Farm, Beaudesert, Queensland.
1983-1984	Field Agent National Mutual Life Association
1980-1983	Production Manager Rice's Aussie Bee Farm, Beaudesert, Queensland.
1978-1979	Honeybee Technologist Rice's Aussie Bee Farm, Beaudesert, Queensland.

Selection Criteria

Pre-requisite:

1.0 A PhD degree in molecular biology

I am about to submit a thesis for a Doctorate in Philosophy for which the research subject is entirely molecular biological in nature. The thesis is entitled ÒThe Molecular Taxonomy of Two Microsporidia.Ó The impending completion of my Doctorate meets the terms of the fellowship in that I have not had more than 3 years of relevant post-doctoral experience.

2.0 Task Areas and Associated Principal Activities

2.1 Proven research ability in molecular biology including molecular genetics

To demonstrate my research ability in both molecular biology and molecular genetics I will summarize laboratory research in recent years.

Bachelor of Science Honours Degree Research Summary

A genomic library was constructed for the fungus *A. nidulans* in the vector Lambda Gem-11. PCR primers were designed and a probe amplified from *A. oryzae* genomic DNA. The PCR-amplified probe contained sequence encoding the three conserved amino acid residues in the *A. oryzae* alkaline protease gene and flanking sequence known to be homologous within the subtilisin family. The *A. nidulans* genomic library was screened using this PCR-amplified probe. Two lambda transformants that potentially contained the putative alkaline protease gene of *A. nidulans* were isolated. Phage genomic DNA was isolated from these transformants and a restriction map of the inserts constructed. The maps suggested that a region of approximately 3 kb, containing two adjacent Xho I fragments, appeared to be in common between the two lambda clones. Southern blot analysis demonstrated that this 3 kb region and a Sac I/Eco RI sub-fragment from within this region were homologous to the PCR-amplified probe. A probe was constructed using this Sac I/Eco RI fragment.

Total RNA was isolated from the mycelium of five strains of *A. nidulans*. These strains exhibit a known pattern of protease expression when grown under different nutrient limiting and non-limiting conditions. Dot blot analyses of total RNA with the Sac I/Eco RI probe exhibited hybridization patterns consistent with the pattern of protease expression known to occur for the five mutant strains of *A. nidulans*. These results provided supportive evidence that all or part of the putative alkaline protease gene from *A. nidulans* had been isolated.

The nucleotide sequence of the *A. nidulans* alkaline protease gene was determined. The gene was found to be composed of four exons separated by three introns.

Doctorate Research Summary

The microsporidia are a very ancient group of obligate parasitic protists. They have an extensive host range including members of the phyla Arthropoda and Chordata. The microsporidia are known to have unusual cytological and molecular characteristics and have ribosomes and ribosomal RNAs

(rRNA) that are prokaryotic in size. Morphology, life cycle and host specificity studies of a few microsporidia have provided the necessary information for the taxonomic classification of microsporidia. However, there is considerable debate as to the accuracy of this taxonomic classification. The subject of this thesis is to determine the true taxonomic classification of the microsporidia. For this study the complete ribosomal operon was sequenced for one of two species of microsporida while the internal transcribed spacer and the large subunit were sequenced for the other species of microsporidia.

In order to undertake this research, several new techniques were developed. Because of the obligate intracellular parasitic nature of microsporidia the only phase of the life cycle from which genomic DNA can reliably be extracted is during the resting or spore phase. At this stage of the parasite's life cycle, it is enclosed in an extremely tough proteinaceous coat. This resting or spore stage allows the organism to survive in the environment while transferring from its dying host to a new host. The spore is ingested and germinates in response to host-specific chemical and ionic stimuli or otherwise it passes out with the feaces and again waits to be ingested by a new host. To obtain the high molecular weight genomic DNA required for this research, it was necessary to design protocols that encompassed both germination and DNA isolation for each species of microsporidians. The protocol for each species was different as the specific stimuli to trigger germination for both species of microsporidians was found to be different.

Secondly, as the non-transcribed spacer was to be sequenced it was necessary to develop a PCR technique that reliably amplifies fragments greater than 5kb, allowing for the use of conserved sequences within the ribosomal DNA. Kits are now available for expanded PCR. However, these kits were not available at the time this research was undertaken. Research reports published in 1993-94 demonstrated the potential for expanded PCR using lambda clones. In conjunction with these reports I developed a protocol that allowed for the amplification of fragments containing the non-transcribed spacer from genomic DNA. Eventually, I was able to amplify the entire ribosomal operons of the two microsporidians under study directly from genomic DNA. Furthermore, I demonstrated the usefulness of this technique by amplifying the entire ribosomal operon from the yeast *Cryptococcus neoformans* and then by amplifying entire plasmids containing inserts. This technique is potentially useful for site-directed mutagenesis of plasmid inserts.

I also have experience in cloning of large fragments, site-directed deletion, dye primer sequencing and dye terminator sequencing from clones and PCR products, together with sequence analysis using a number of software packages.

The results from my Doctorate research will be shortly available via my thesis and journal articles.

3.0 Professional/Technical Skills and Experience

3.1 Experience in constructing genome libraries

As outlined in (2.1) above, I have experience with constructing genomic libraries. As part of my honours degree research program I constructed a genomic library for the fungus *Aspergillus nidulans* in the vector Lambda Gem 11. The titre of this library was approximately 8 times that required for full representation of the *A. nidulans* genome. Additionally, isolated clones were mapped for a range of restriction sites.

3.2 Experience in DNA manipulation and mutagenesis

As outlined in (2.1) above, I have experience in DNA manipulation and mutagenesis. Technical skills include:-

- PCR both standard and extended
- Cloning in either plasmid or phage vectors
- Site-directed deletions by restriction digest and exonuclease digestion
- Insertion mutations by restriction and synthetic fragment insertion
- Site-directed mutations using extended PCR

3.3 Experience in communicating on a professional level

3.3.1 Scientific Communication.

Doctorate of Philosophy Thesis, 1996.
The molecular taxonomy of two microsporidians.
Principal Supervisors Dr. D. Anderson and Dr. P. Cooper

Honours Thesis, 1992.
The isolation of a putative alkaline protease gene from *Aspergillus nidulans*.
Supervisor Dr. M. E. Katz.

Isolation of an alkaline protease gene and regulation of extracellular protease production in *Aspergillus nidulans*. (1994) Gene 150, 287-292.
Margaret E. Katz, Robert N. Rice, Pam K. Flynn.

Paper Presented to the 17th Fungal Genetics Conference, Asilomar, California, 1993.
Molecular and genetic analysis of extracellular protease production in *Aspergillus nidulans*.
Margaret E. Katz, Robert N. Rice, Pam K. Flynn, and Brian F. Cheetham.

Paper Presented to the Lorne Genome Conference, Lorne, Victoria, 1994.
Regulation of extracellular protease production in *Aspergillus nidulans*.
Margaret E. Katz, Pam K. Flynn, Amir Masoumi, Robert N. Rice, Patrica van Kuyk, and Brian F. Cheetham.

3.3.2 Commissioned Survey and Disease Reviews.

A survey commissioned by The Ministry of Foreign Affairs and Trade, Wellington, New Zealand. March 1996.
A survey of blister beetles in honey bee colonies on Guadalcanal, Solomon Islands.
Robert N. Rice and G. Murray Reid.

A review and risk analysis commissioned by the Ministry of Agriculture Regulatory Authority, Wellington, New Zealand. April 1996.
European foulbrood, an exotic honey bee disease to New Zealand: An epidemiological review and risk analysis.
Robert N. Rice.

3.3.3 Industry Publications.

Disease Facts Part 1: *Nosema apis* a pathogen of honey bees.
Beefax Vol 1:1(1995), Ministry of Agriculture - Quality Management.
Taraunga, New Zealand.
Robert N. Rice

Disease Facts Part 2: *Nosema apis* a pathogen of honey bees.
Beefax Vol 1:2 (1995), Ministry of Agriculture - Quality Management.
Taraunga, New Zealand.
Robert N. Rice.

European Foulbrood a pathogen of honey bees.
Beefax Vol 1:4 (1996), Ministry of Agriculture - Quality Management.
Taraunga, New Zealand.
Robert N. Rice.

Undoing the biological zipper.
Beefax Vol 1:5 (1996), Ministry of Agriculture - Quality Management.
Taraunga, New Zealand.
Robert N. Rice.

3.4 Experience in the use of computers and database analysis.

My experience in using computers and database analysis is quite extensive. I am fluent in the use of commercial software such as Windows, Word for Windows, Excel (Microsoft) and WordPerfect for Windows (WordPerfect Corporation).

For research purposes I have used GCG (Genetic Computer Group, Inc.), PAUP - Phylogenetic analysis using parsimony (Swofford, D.L.), RNA_D2 (Dorisse-Perochon, J. and Michot, B.), DCSE - Dedicated Comparative Sequence Editor (De Rijk, P.) and CARD - A computer program for drawing RNA secondary structure models (Winnepenninckx, B. *et al.*), SEAVIEW and PHYLO_WIN - two graphic tools for sequence alignment and molecular phylogeny.

In addition to the use of the above mentioned software, I am fluent in the use of the internet including the World Wide Web, Gopher and FTP (File Transfer Protocol).

4.0 Personal Attributes

4.1 Proven ability to interact cooperatively and harmoniously with a variety of staff members and collaborators.

I demonstrate my abilities to interact cooperatively and harmoniously with others in two ways.

With the assistance of three employees I was directly responsible for the maintenance of 1,000 honey producing colonies, 7,000 mating colonies and 750 support colonies used in the maintenance of the mating colonies.

Secondly, as Apiculture Service Manager (South Island) for the Ministry of Agriculture - Quality Management, I work as part of a team, the National Apicultural Business Unit (NABU). Within this team I am responsible for the delivery of apiculture services to Government and beekeeping industry clients within the South Island of New Zealand. Major components of this role include: co-ordination and contribution to design of surveillance programmes for detection of exotic bee diseases such as European foulbrood disease, *Varroa, Tropilaelaps* and *Acarapis* mites; apiculture training for staff; implementation of response plans for exotic bee diseases; design and implementation of an endemic (American foulbrood) disease control programme; extension activities with individual beekeepers to help them control disease and improve the profitability of their operations; export certification; providing technical advice to Government on apicultural issues; providing consultant services; implementing provisions of the Apiaries Act and related legislation. Within this position I have additional technical roles as a bee disease epidemiologist and researching technical improvements to surveillance and disease response systems. As demonstrated by the broad range of my duties it is necessary that I have the ability to interact co-operatively and harmoniously with a large number of people from diverse backgrounds both occupational and ethnic.

4.2 Demonstrate the ability to work effectively without close, direct supervision.

As outlined in (4.1) I have clearly demonstrated my abilities in working effectively without close, direct supervision. This ability was necessary both in my occupation as production manager for A "Rice's Aussie Bee Farm" and in my current position as Apiculture Service Manager.

4.3 Ability to work in accordance with EEO, OH&S and Industrial Democracy principles.

I personally have a commitment to working in and support of the Equal Employment Opportunities (EEO) environment. I am fully aware of the principles of Occupational Health and Safety (OH&S) and Industrial Democracy. The Ministry of Agriculture operates entirely within this environment and under these principles.

5.0 Commitment

5.1 Demonstrate commitment to a high level of personal performance and the provision of quality outcomes.

High levels of personal performance and quality outcomes are the corner stone of my philosophy of life. I am a highly self-motivated individual and have shown a high degree of initiative. A summary of my life's achievements demonstrates these qualities. As the production manager of "Rice's Aussie Bee Farm", a highly successful and internationally recognized company, it was my responsibility to meet production deadlines, fulfilling the needs of clients both nationally and internationally. This dedication to the clients' needs generated on-going business from clients over many years. As a self-employed, commissioned-based field agent for the National Mutual Life Insurance Company, my income was governed by my ability to prospect for and generate sales of products marketed by National Mutual. In my first year as a field agent I received an award for meeting of sales goals set by National Mutual. At age 28 I took it upon myself as a married person with children to further my education. This education process has encompassed the completion of a science degree, honours degree and currently a doctorate. In order for me to undertake my Doctorate it was necessary for me

to apply for and be awarded a research grant from the Honey Bee Development and Research Council.

5.2 Ability to adapt to changes in procedural demands in the course of a project.

I would have to say that adaptability is my middle name. In the course of this application I have demonstrated my ability not only to adapt to changes within a specific field but have also demonstrated my ability to adapt to complete changes in fields of pursuit.

Referees

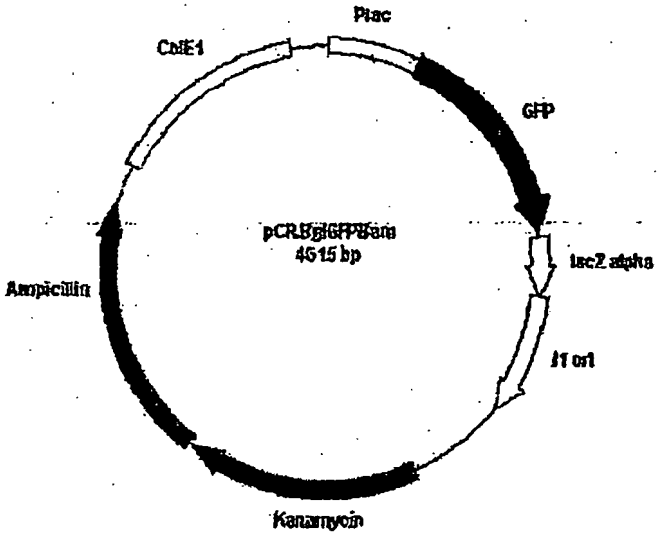
Dr D. Anderson, CSIRO Division of Entomology, GPO Box 1700, Canberra, ACT, 2601.

Dr P. Cooper, Botany and Zoology Department, Australian National University, Canberra, ACT, 0200.

Dr P. Keese, CSIRO Division of Plant Industries, GPO Box 1600, Canberra, ACT, 2601.

Dr A. Gibbs, Research School of Biological Sciences, Australian National University, Canberra, ACT, 0200.

EXHIBIT 3



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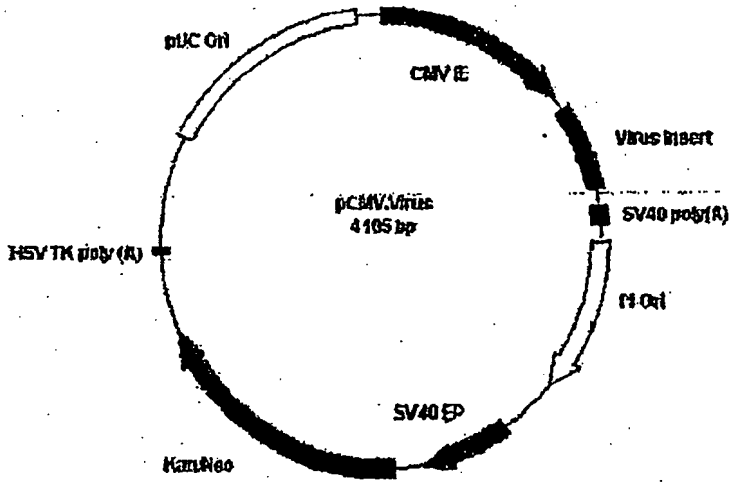

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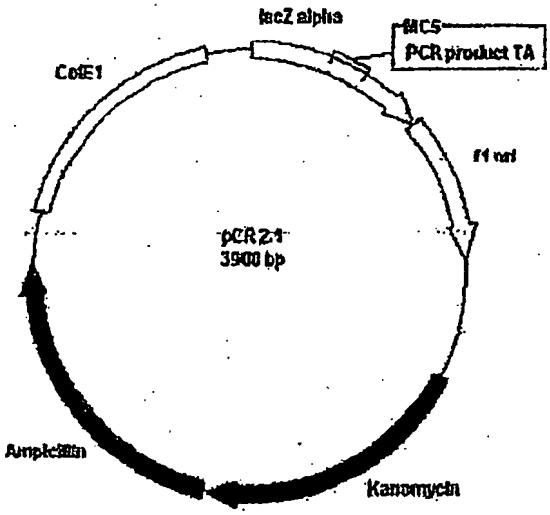

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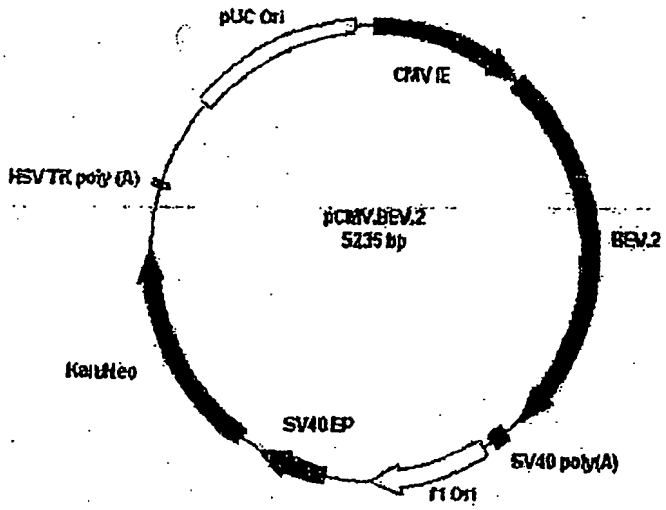

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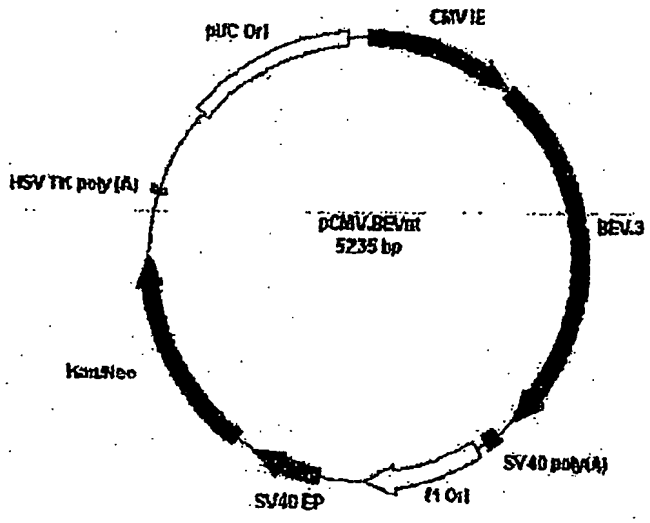

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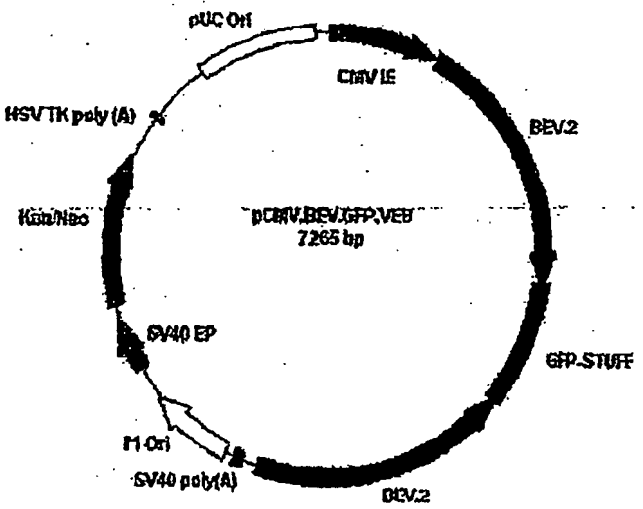

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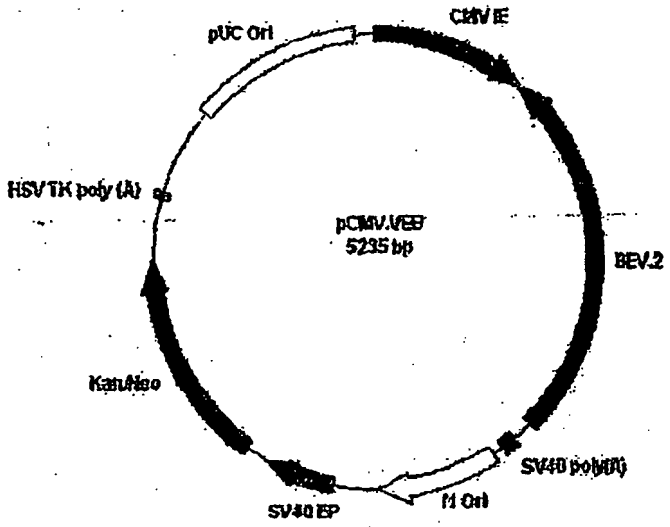

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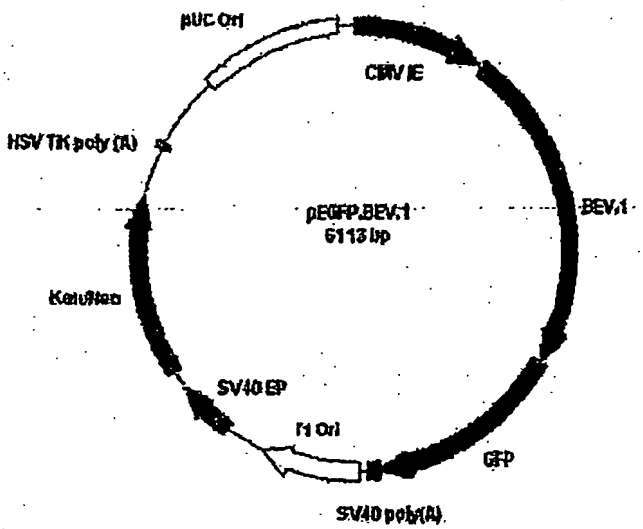

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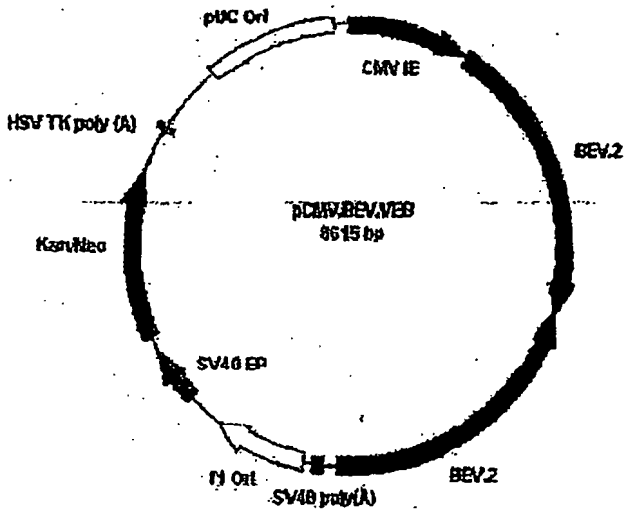

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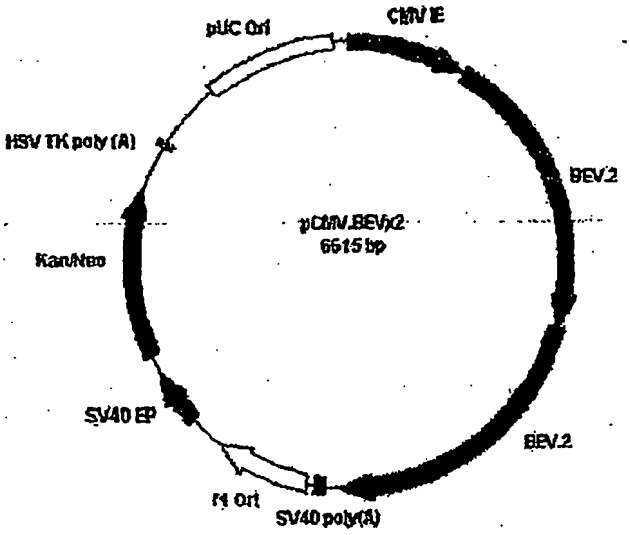

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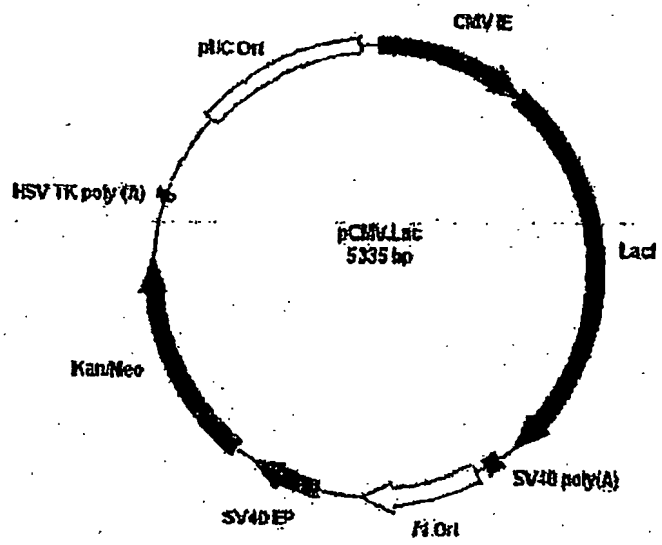

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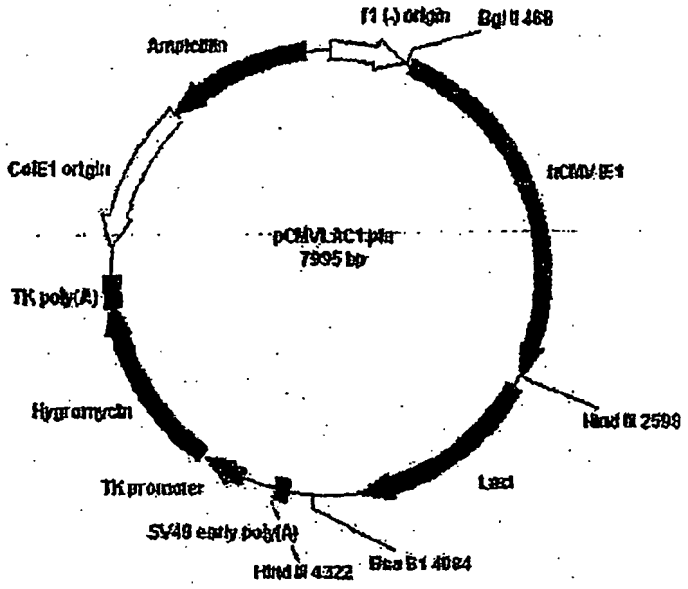

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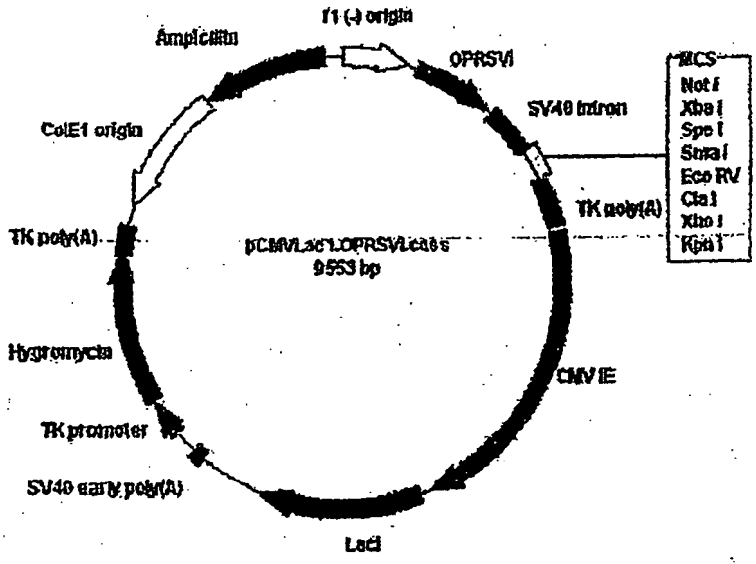

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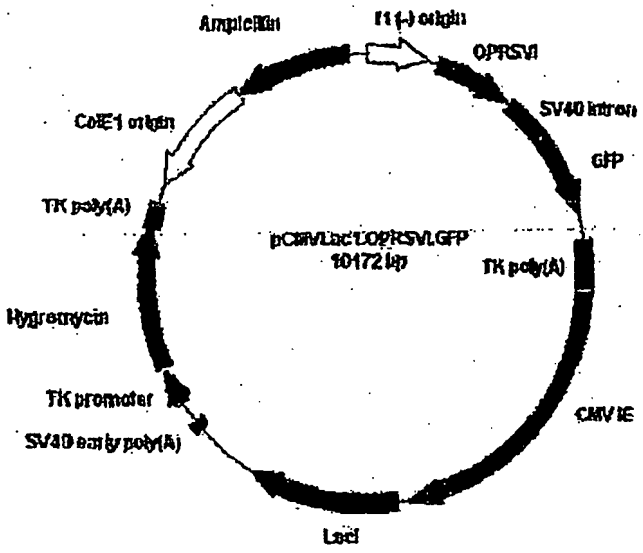

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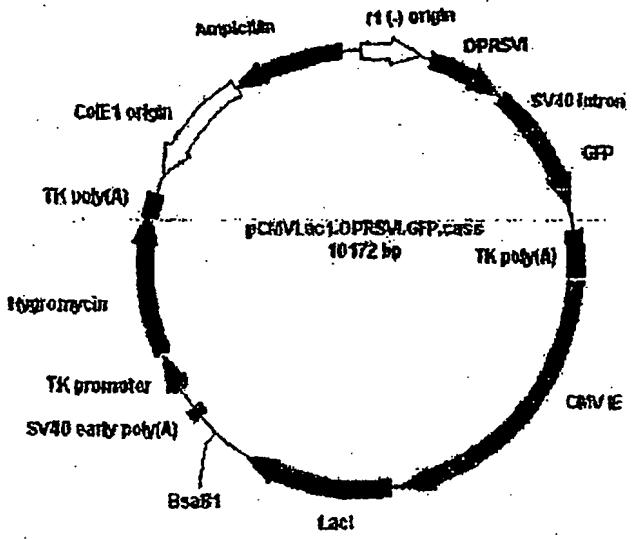

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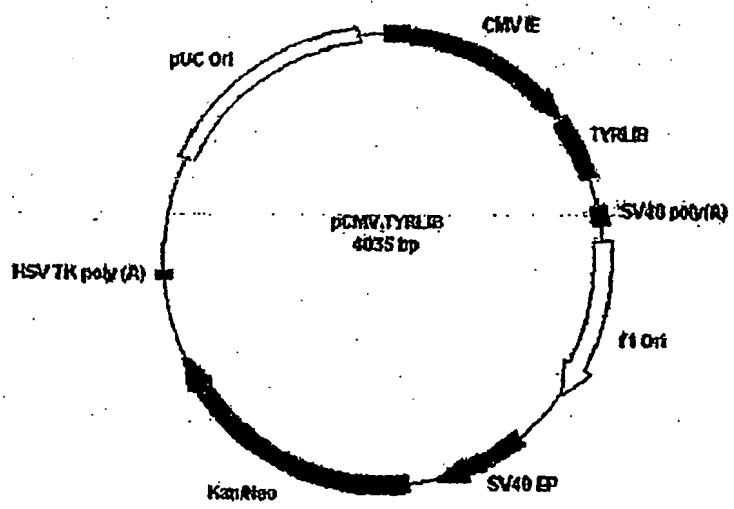

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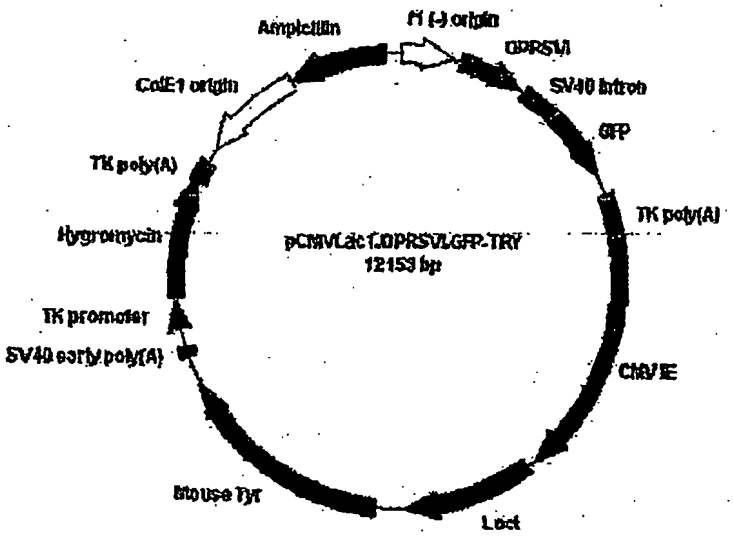

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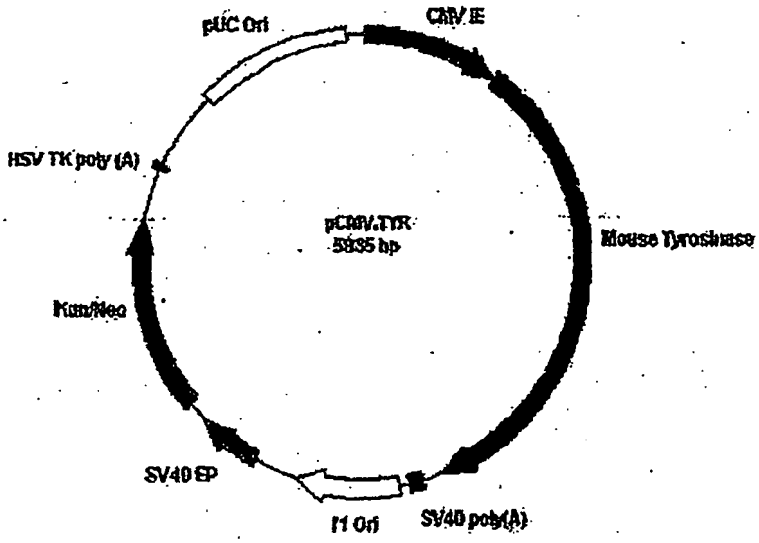

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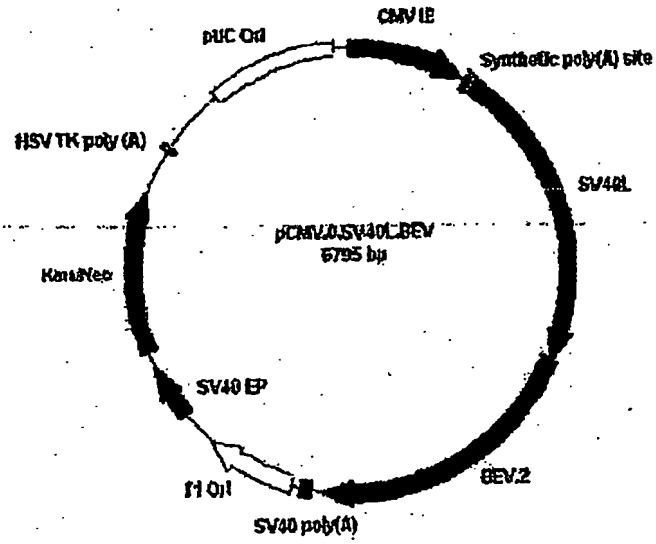

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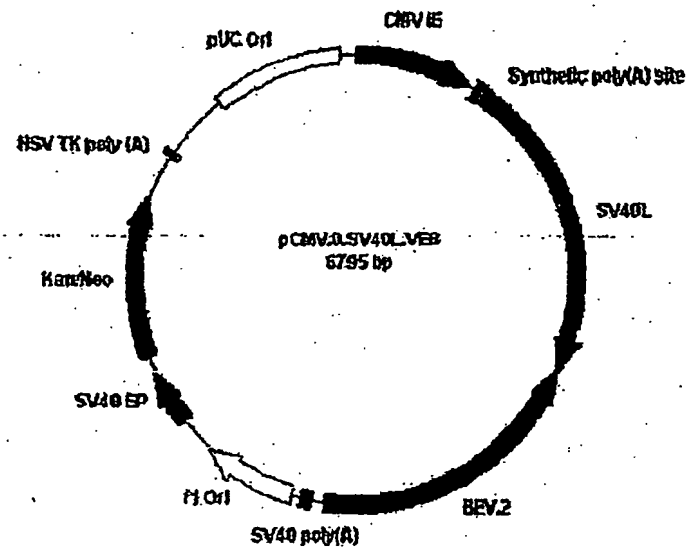

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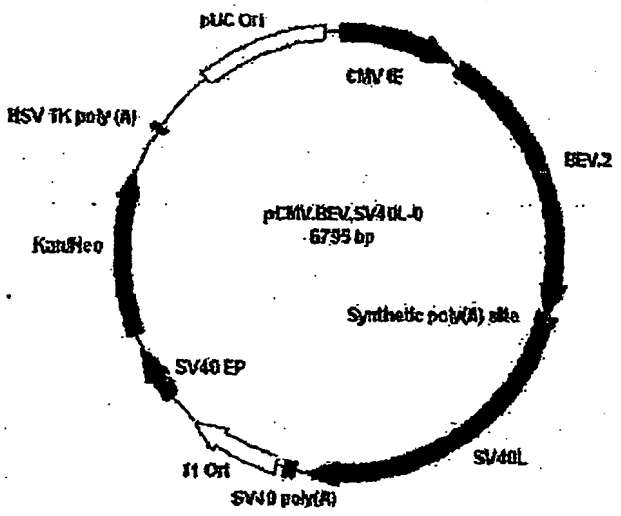

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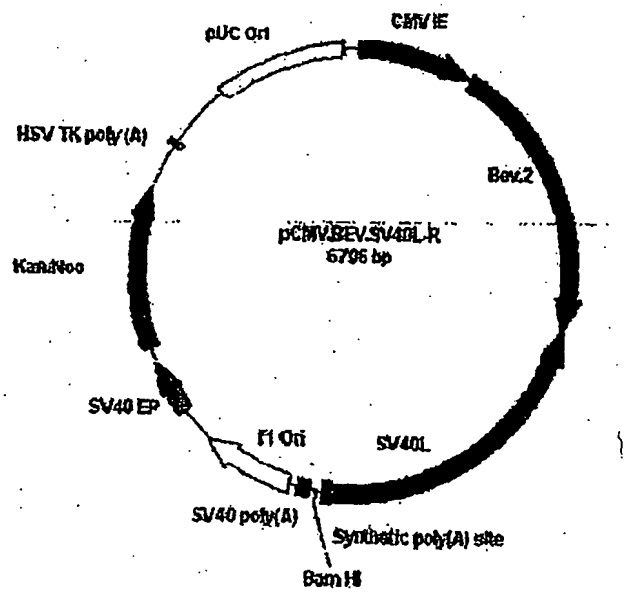

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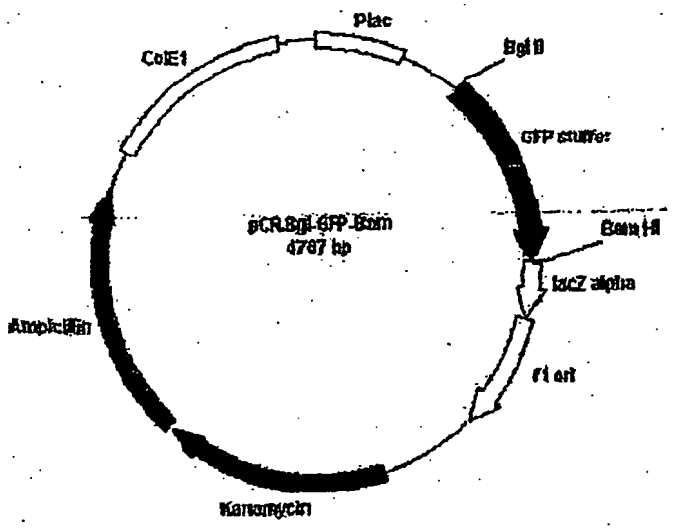

Author: PCBGFPBA.PLA
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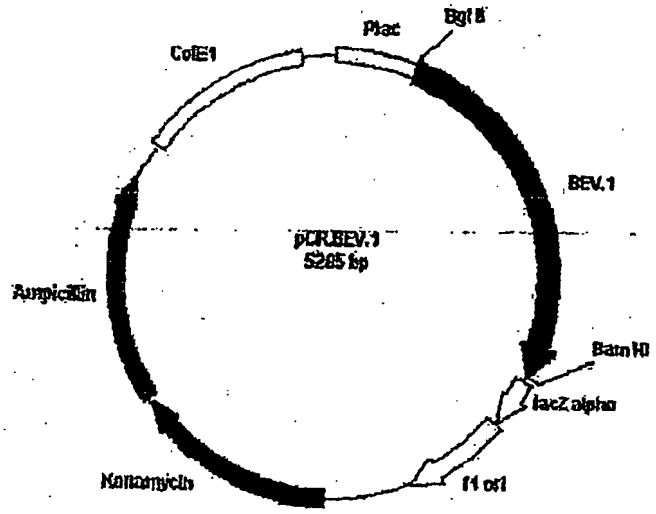

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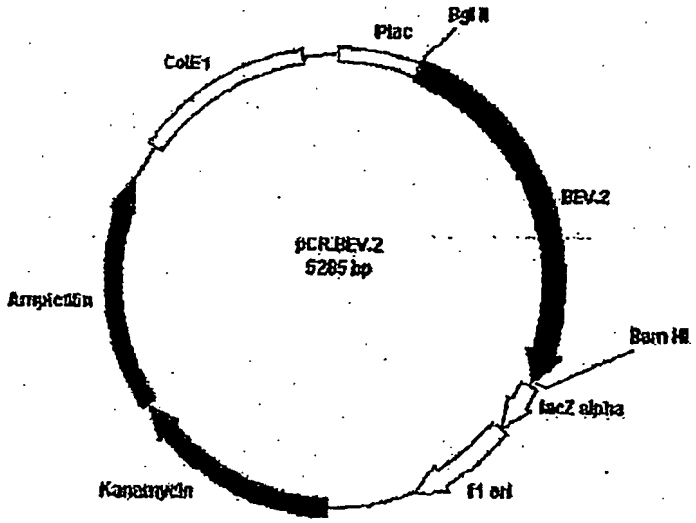

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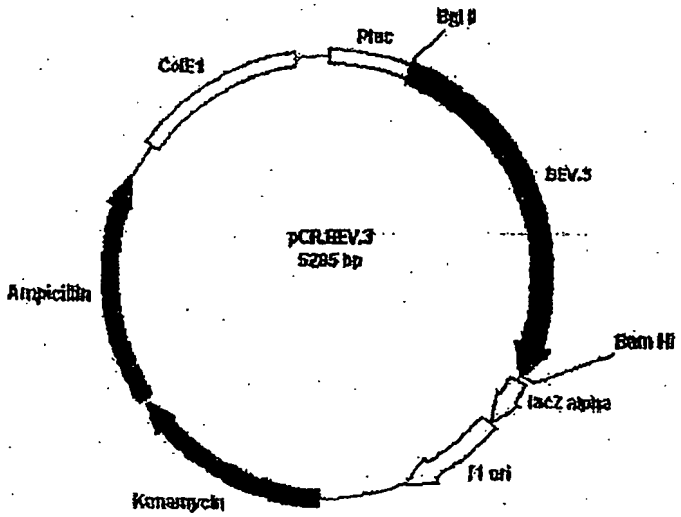

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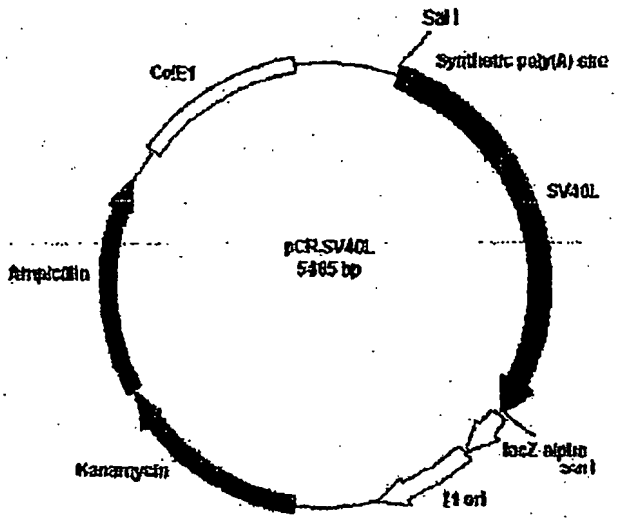

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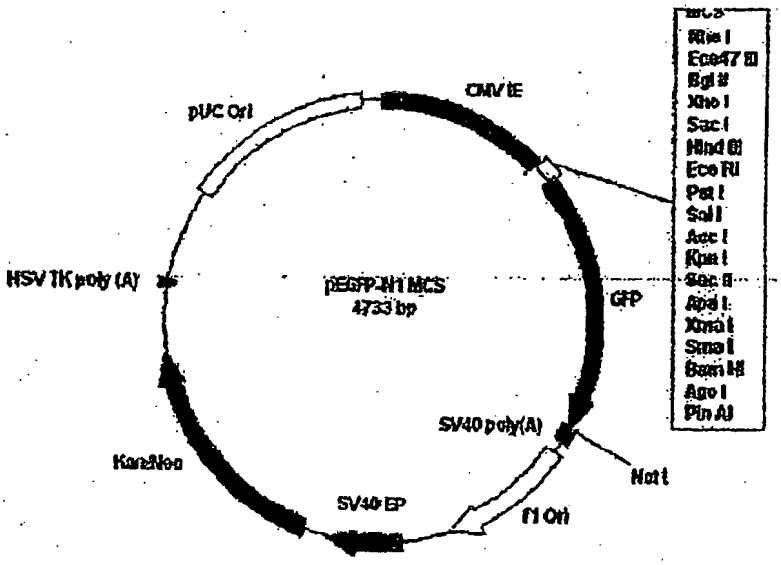

Author: Robert Rice
Date: 22/1/98
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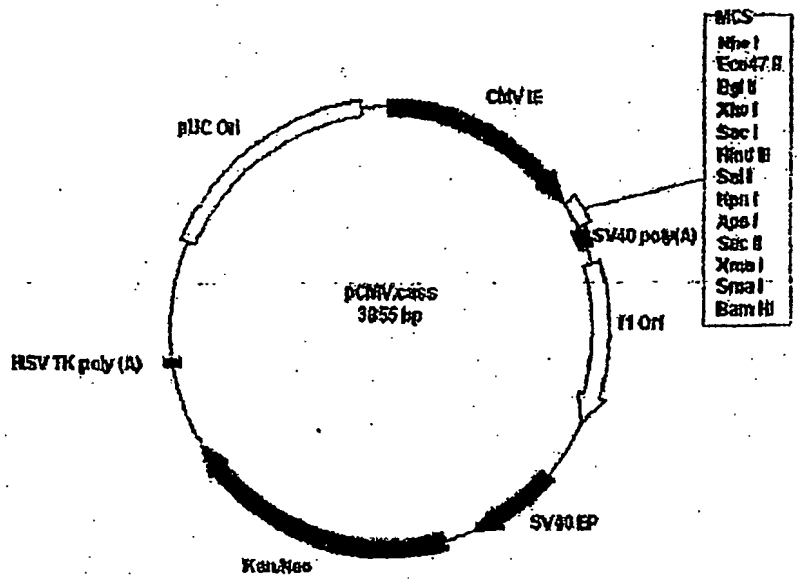

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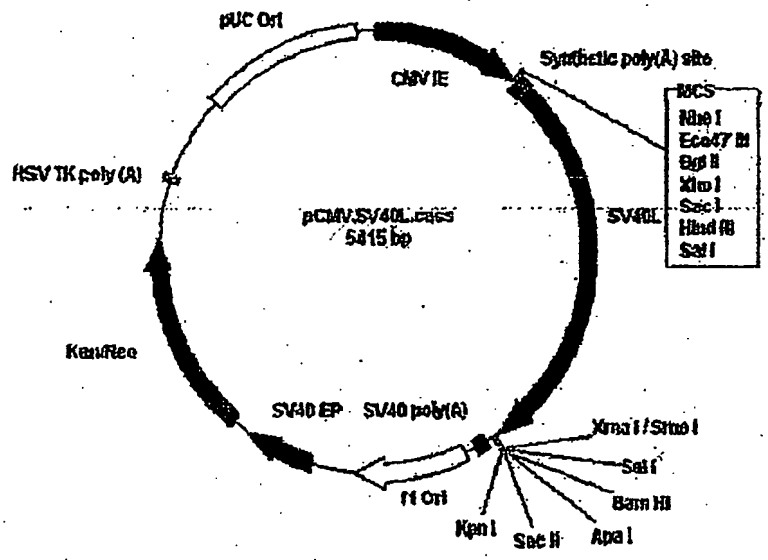

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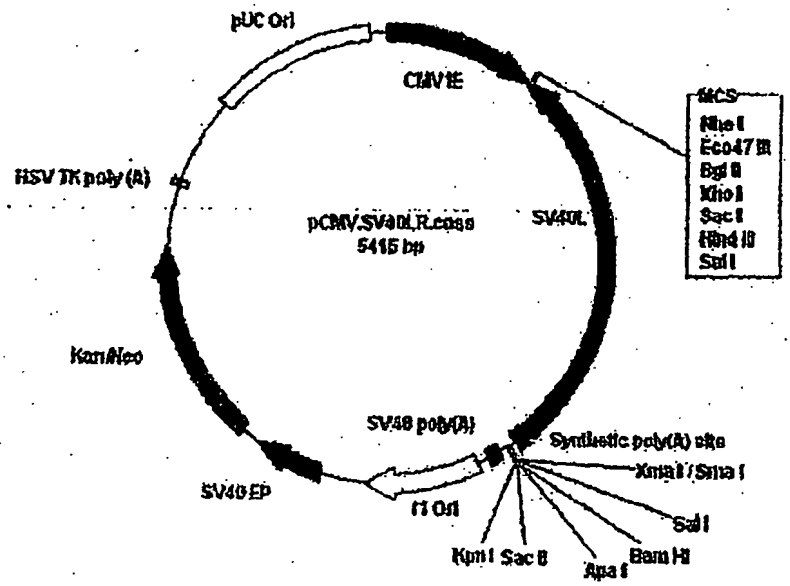

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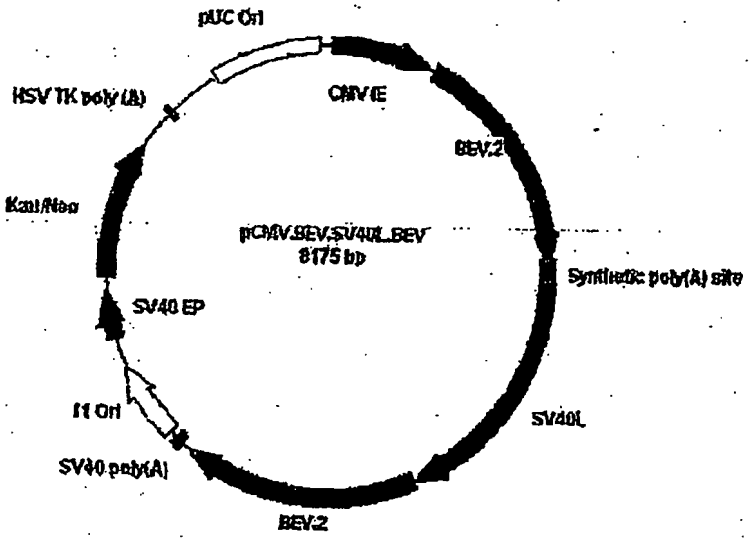

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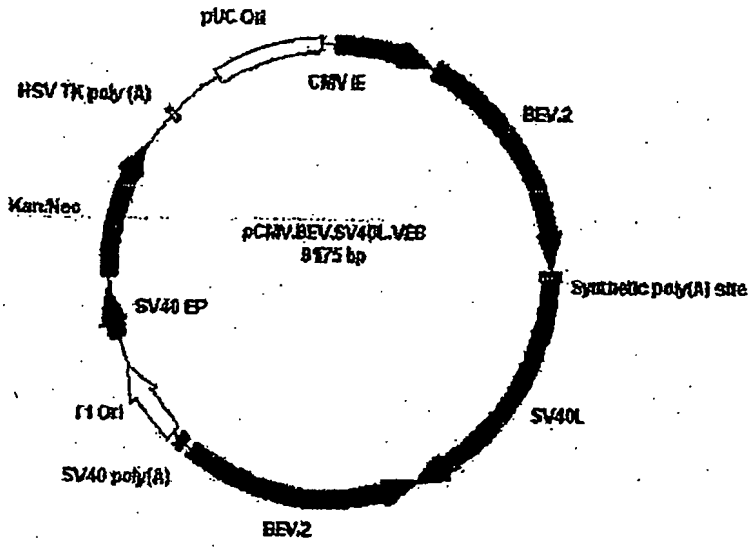

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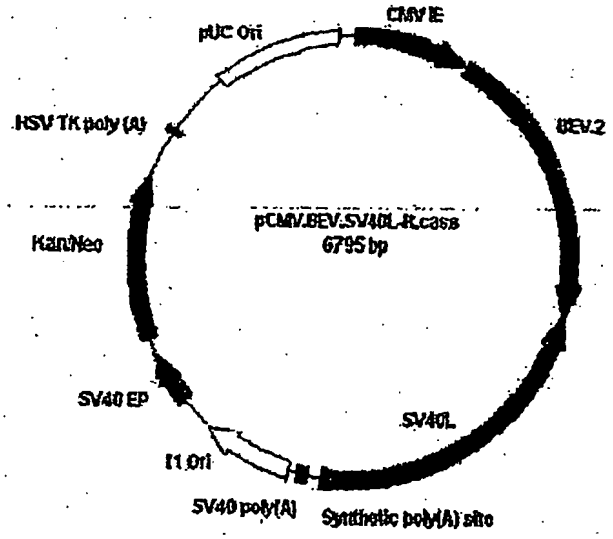

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